Ketogenic Therapy as an Adjuvant for Malignant Glioma: Impacts on Anti-Tumor Immunity

by

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ABSTRACT

Malignant brain tumors are devastating despite aggressive treatments such as surgical resection, chemotherapy and radiation therapy. The average life expectancy of patients with newly diagnosed glioblastoma is approximately 15 months. One novel therapeutic strategy involves using a ketogenic diet (KD) which increases circulating ketones and reduces circulating glucose. While the preclinical work has shown that the KD increases survival, enhances radiation and alters several pathways in malignant gliomas, its impact on the anti-tumor immune response has yet to be examined. This dissertation demonstrates that mice fed the KD had increased tumor-reactive innate and adaptive immune responses, including increased cytokine production and cytolysis via tumor-reactive CD8+ T cells. Additionally, we saw that mice maintained on the KD had increased CD4 infiltration, while T regulatory cell numbers stayed consistent. Lastly, mice fed the KD had a significant reduction in immune inhibitory receptor expression as well as decreased inhibitory ligand expression on glioma cells, namely programmed death receptor -1 (PD-1) and its ligand programmed death receptor ligand -1 (PD-L1). Further, it is demonstrated that the ketone body beta-hydroxybutyrate (BHB) reduces expression of PD-L1 on glioma cells *in vitro* suggesting it may be responsible in part for immune-related changes elicited by the KD. Finally this dissertation also shows that the KD increases the expression of microRNAs predicted to target PD-L1 suggesting a potential mechanism to explain the ability of the KD to modulate immune inhibitory checkpoint pathways. Taken together these studies shed important light on the mechanisms underlying the KD and provide additional support for its use an adjuvant therapy for malignant glioma.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

Figure Page

LIST OF ABBREVIATIONS

ACA	Acetoacetate
AMPK	Adenosine Monophosphate-Activated Protein Kinase
ATP	Adenosine Triphosphate
BCNU	1,3-Bis(2-Chloroethyl)-1 nitrosourea
BHB	Beta Hydroxybutyrate
CAIX	Carbonic Anhydrase 9
CR	Caloric Restriction
CTLA4	Cytotoxic T-Lymphocyte-Associated protein 4
COX-2	Cyclooxygenase-2
DNA	Deoxyribonucleic Acid
EGFR	Epidermal Growth Factor Receptor
FDG-PET	Fluorodeoxyglucose Positron Emission Tomography
GBM	Gliobastoma multiforme
HDAC	Histone Deacetylase
HIF-1α	Hypoxia Inducible Factor-1 alpha
IDO	Indoleamine 2,3 Dioxygenase
IF	Intermittent Fasting
IFN-γ	Interferon Gamma
IGF-1	Insulin Growth Factor-1
IL	Interleukin
JAK	Janus Kinase
KC	KetoCal®
KD	Ketogenic Diet

LAG-3	Lymphocyte Activation Gene-3
LCMV	Lymphocytic Choriomeningitis Virus
MAPK	Mitogen-Activated Protein Kinase
MCT	Medium Chain Triglycerides
miRNA	MicroRNA
mTOR	Mammalian Target of Rapamycin
NF-κB	Nuclear Factor Kappa B
NK Cells	Natural Killer Cells
PD-1	Programmed Death Receptor-1
PD-L1	Programmed Death Receptor-1 Ligand
PDK2	Pyruvate Dehydrogenase Kinase Isoform 2
PI3K	Phosphatidylinositol-3 Kinase
PTEN	Phosphatase and Tensin Homolog
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
STAT3	Signal Transducer and Activator of Transcription 3
TIL	Tumor-Infiltrating Lymphocyte
TNF	Tumor Necrosis Factor
TMZ	Temozolomide
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

INTRODUCTION

Human malignant glioma is a uniformly fatal disease due, in part, to the limitations of currently available treatments which include surgery, chemotherapy and radiation therapy. Average survival of patients with glioblastoma multiforme (GBM) is 1.5 years, and tumors of the central nervous system are the most common solid tumor in the pediatric population. It is therefore of paramount importance that new therapeutic strategies for brain cancer patients be developed, especially those that can enhance the efficacy of current treatment options without damaging normal brain tissue. Advances in our understanding of the biology of these tumors has led to an increase in the number of targeted therapies in preclinical and clinical trials [1]. While these therapies may prove somewhat effective, the heterogeneity of this tumor often precludes the targeted molecules from being found on all cells in the tumor thus reducing the efficacy of these treatments. In contrast, one trait shared by virtually all tumor cells is altered metabolism.

Tumor Metabolism

Alterations in the metabolism of cancer cells, what we now call the “Warburg effect” or aerobic glycolysis, was first described by Otto Warburg in 1927 [2]. Cancer cells use glycolysis to provide energy and biomolecules regardless of the availability of oxygen. This results in the production of fewer ATP molecules per molecule of glucose, and thus tumor cells require large amounts of glucose. This shift towards increased glycolytic flux in the cytosol and away from the tricarboxylic acid cycle and oxidative

phosphorylation in the mitochondria occurs very early in tumorigenesis. This allows for rapid cell proliferation even under conditions of hypoxia and in the presence of dysfunctional mitochondria. Since Warburg's discovery, metabolism has been of interest in the cancer field but it often seemed overshadowed by discoveries of oncogenes, tumor suppressor genes, growth factor pathways, molecular subtypes of cancers, etc. There is a resurgence of interest in metabolism as a central theme in cancer, and we continue to find that metabolic pathways intersect and often regulate key components of tumor initiation, progression and therapy response [3, 4]. In fact, altered metabolism itself has been referred to as a hallmark of cancer [5, 6] in addition to being involved in virtually all of the cancer hallmarks described in the seminal paper by Hanahan and Weinberg [7] (Figure 1, [8]).

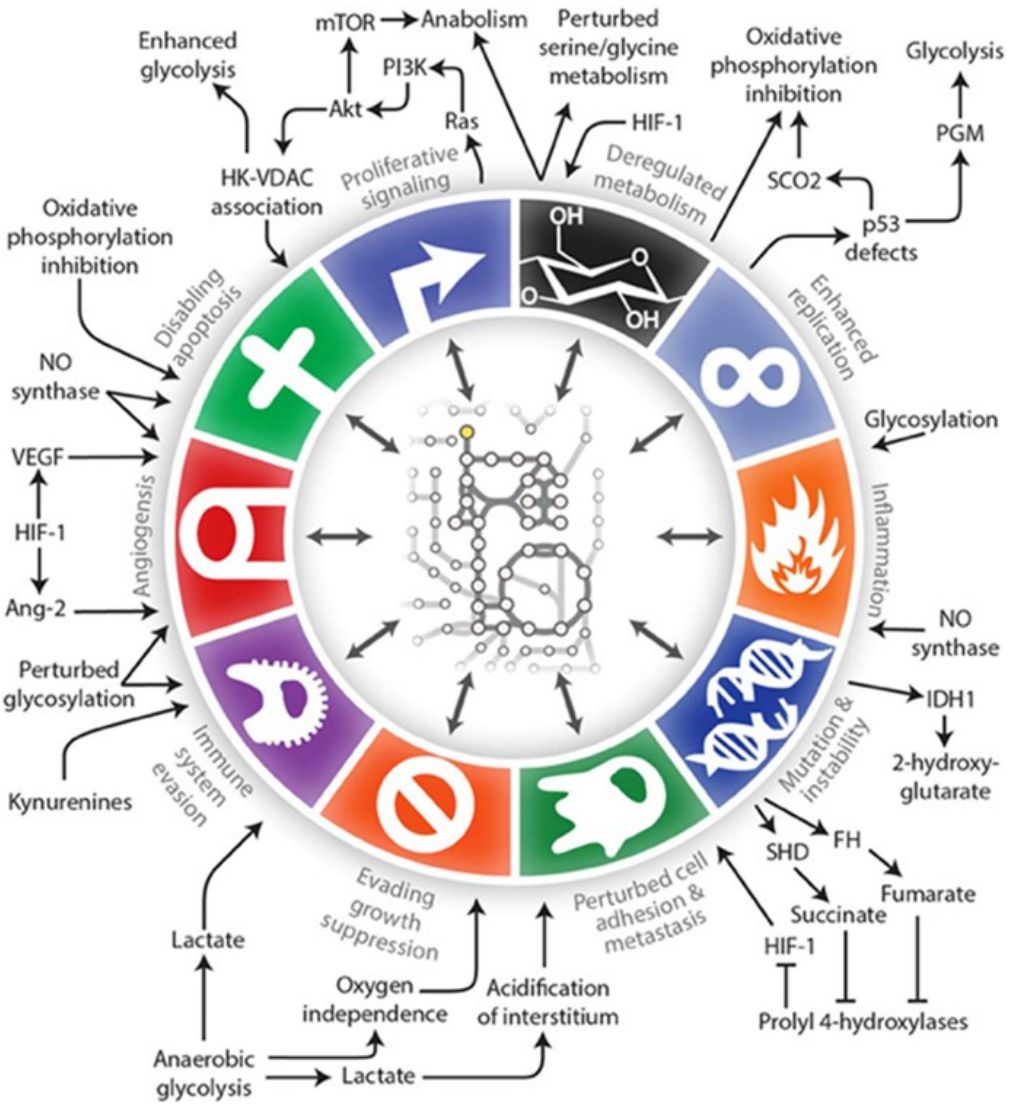


Figure 1: **An illustration of the interconnections between tumor metabolism with Hanahan and Weinberg's Hallmarks of Cancer** [8].

The term “metabolic remodeling” has been used to describe metabolic changes that can occur in cancer cells [9], and oncogene associated pathways are now known to intersect with, and alter metabolic pathways. For example, the tumor suppressor protein p53 which plays a pivotal role in the cellular responses to hypoxia, DNA damage and oncogene activation is now known to regulate glycolysis and assist in maintaining mitochondrial integrity [9]. Another important connection between metabolism and tumor growth is through regulation of c-MYC. Over-expression of c-MYC occurs in a wide variety of cancers including gliomas. C-MYC is a multi-functional transcription factor and the list of its target genes include those involved in both cell proliferation and cell metabolism [10]. In addition to stimulating glycolysis, c-MYC has been found to activate glutaminolysis and lipid synthesis from citrate [9].

With the advent of molecular analyses, studies of growth factor pathways seemed to overshadow the influence of metabolism on cancer growth. Over-activation of the stress responsive PI3K/AKT signaling pathway is typical in many cancers and often due to activation of growth factor signaling pathways involved in glioma growth such as platelet-derived growth factor, epidermal growth factor and insulin growth factor. We now know that these growth factor pathways are intertwined with metabolic signally pathways [11-14]. PI3K/AKT signaling has been closely linked to metabolism and under low glucose conditions results in rapid tumor cell death [15].

Another important “hub” linking metabolism and cancer is hypoxia-inducible factor 1 (HIF-1). HIF-1 expression is activated by hypoxia, which is typically found in high grade gliomas and other cancers. HIF-1 is a heterodimeric transcription factor that induces the transcription of a variety of genes involved in angiogenesis (vascular endothelial growth factor (VEGF) and other cytokines) in an attempt to improve tissue perfusion. This results in the formation of abnormal blood vessels that can increase

inflammation and edema in brain tumors, as well as induction of the transcription of a variety of genes that promote invasion, migration and tumor growth [16, 17]. In addition to specific actions that relate to the tumor cell's response to oxygen availability, HIF-1 interacts with the PI3K/AKT signaling path to act as a regulator of cancer metabolism, proliferation and glycolysis [12, 17-19]. HIF-1 may, at least in part, provide the molecular basis for the Warburg effect by "reprograming" cellular metabolism in response to oxygen availability [12, 20]. HIF-1 also is a central figure in alterations to the tumor microenvironment which not only affects tumor cell growth, but also response to therapy [15, 17, 21]. It also affects the activation of nuclear factor – kappa B (NF-κB), a transcriptional activator that is central to the regulation of various signal transduction pathways and to transcriptional activation events that mediate inflammation, cell proliferation, cell migration, and angiogenesis.

The molecular background of a tumor cell can also affect the regulation of the pathways described above. Loss of function of phosphatase and tensin homolog (PTEN) or mutation of p53 also increase HIF-1, as does the accumulation of reactive oxygen species (ROS). ROS are multi-faceted effector molecules involved in numerous cellular pathways, including those regulating autophagic/apoptotic responses to genotoxic stress, hypoxia and nutrient deprivation. Cancer cells often have increased levels of ROS [22] and they have been implicated in angiogenesis induction and tumor growth through the regulation of VEGF and HIF-1 [23].

It is clear that cancer cell metabolism is far more complex than originally thought. A number of cancer associated mutations affect metabolism and defects in mitochondria are seen in cancer that also link metabolism with cancer initiation and progression. Although some of these interactions are mentioned above, in-depth discussions of all of the interactions that occur between cancer and metabolism are beyond the scope of this

dissertation and the reader is referred to a number of reviews on these subjects [5, 6, 22, 24, 25]. The fact that metabolic dysregulation is seen in virtually all tumor cells has led to suggestions that a promising therapeutic strategy may be to exploit this feature. One potential way to achieve this goal is through the use of the therapeutic ketogenic diet (KD) or physiologically similar methods, such as caloric restriction or intermittent fasting.

The Ketogenic Diet

The ketogenic diet (KD) is more correctly referred to as “metabolic therapy” rather than a “diet”. This high-fat low protein/carbohydrate diet is used to treat refractory epilepsy in children, and more recently in some adults [26, 27]. The diet is not without side effects; however, these are typically readily managed when the patient has appropriate supervision by a registered dietitian skilled in its use. The KD has been shown to have neuroprotective effects and there are now studies to determine its efficacy for the treatment of a number of neurological disorders, including Alzheimer’s disease, traumatic brain injury, amyotrophic lateral sclerosis and autism [28-30]. The KD increases blood ketones and decreases blood glucose by simulating the physiological response to fasting, thus leading to high rates of fatty acid oxidation and an increase in the production of acetyl coenzyme A (acetyl-CoA). When the amount of acetyl-CoA exceeds the capacity of the tricarboxylic acid cycle to utilize it, there is an increase in the production of the ketone bodies β-hydroxybutyrate (BHB) and acetoacetate (ACA), which can be used as an energy source in the normal brain [31-35]. Since normal cells readily use ketones as an alternate energy source, they are unlikely to be adversely affected by reduced glucose. In contrast, the metabolic alterations found in cancer cells

are generally thought to reduce their ability to be “flexible” regarding their primary energy source, and thus they require glucose [36-42]. This lends credence to the theory that reduction in glucose that results from the ketogenic diet essentially “starves” the tumor cells and inhibits their growth [43-49]. Thus when used as a therapy, the KD may take advantage of the Warburg effect.

In addition to the effects mediated by glucose reduction, more recent work in cancer research has shown that the ketogenic diet can exhibit anti-tumor effects even in the absence of glucose reduction. We demonstrated the effect of adding ketones to media containing glucose *in vitro* using the AO2V4 cell line [50]. This cell line was derived from a recurrent human glioblastoma and is grown in Waymouth’s MAB 87/3 media containing 28mM glucose and supplemented with 20% fetal calf serum. When 5mM BHB plus 5mM ACA was added to complete media, cell growth was significantly inhibited. When 1,3-bis(2-chloroethyl)-1 nitrosourea (BCNU, carmustine), one of the chemotherapeutic agents given to this patient prior to tumor recurrence was used in addition to ketones there was additional growth inhibition compared to either ketones or BCNU alone. Additional work has shown that the ketones themselves exert antitumor effects separate from the effects of reduced blood glucose [50-52]. The ketone BHB has recently been shown to inhibit histone deacetylases (HDACs) which can result in epigenetic suppression of the expression of a variety of genes. Thus, ketones may provide a link between metabolism and tumorigenesis, although the precise nature of these changes is as yet unknown [53-56]. The concept of ketone treatment in the absence of glucose reduction will be explored further in *Chapter 3* of this dissertation.

Preclinical Evidence

The use of metabolic alteration for the therapy of brain tumors has been championed by Seyfried and colleagues. They used the VM [57] and CT-2A [58] mouse tumor models to show that a ketogenic diet (KD), especially when given in restricted amounts, extends survival. D'Agostino and colleagues have added hyperbaric oxygen and exogenous ketone supplementation to demonstrate reduced tumor cell growth and metastatic spread in the VM metastatic tumor model [59, 60]. We used the syngeneic intracranial GL261-luc/albino C57/Bl6 model to demonstrate that caloric restriction was not necessary for the anti-tumor effect of the KD [61], particularly when a 4:1 fat:carbohydrate plus protein formulation is used [50].

The KD and similar diets used as a monotherapy have a pluripotent effect on the growth of tumors both *in vitro* and *in vivo* which may depend, at least in part, on the model system, the specific metabolic intervention and the molecular underpinnings of the tumor itself [48, 60-70]. The striking feature of the work done to date is that alterations in metabolism have a far reaching effect on tumor cells, tumors and the tumor microenvironment. Studies have shown reductions in growth rate as one might expect; however, there are also changes in the formation of reactive oxygen species and oxidative stress [71-73], angiogenesis [38, 43, 62, 74], hypoxia [37, 60, 62], inflammation and peri-tumoral edema [62, 64, 75], metastasis and invasion [60, 65, 68, 70, 76, 77] and the expression of various transcriptional modulators such as NF-κB [62] and c-MYC (unpublished data, Scheck *et al.*).

KD in Combination with Standard Therapies

Although evidence suggests that the KD provides anti-tumor benefits on its own, perhaps the most effective use of the KD is in combination with standard cancer therapies such as radiation and chemotherapy [78]. The KD greatly enhanced survival in a mouse model of malignant glioma when combined with TMZ when compared to either treatment alone (Figure 2) [79]. Using a bioluminescent, syngeneic intracranial model of malignant glioma, the KD was also shown to significantly potentiate the anti-tumor effect of radiotherapy. In fact, 9 out of 11 animals maintained on the KD and treated with radiation had complete and sustained remission of their implanted tumors, even after being switched back to a standard rodent diet (Figure 2) [80]. Allen et al reported similar results when the KD is combined with radiation and chemotherapy in a lung cancer xenograft model [72]. That is, they found decreased tumor growth rate and increased survival. CR and short-term fasting have also been found to be synergistic with radiation and other anti-cancer therapeutics in both preclinical and clinical studies [42, 63, 81-89].

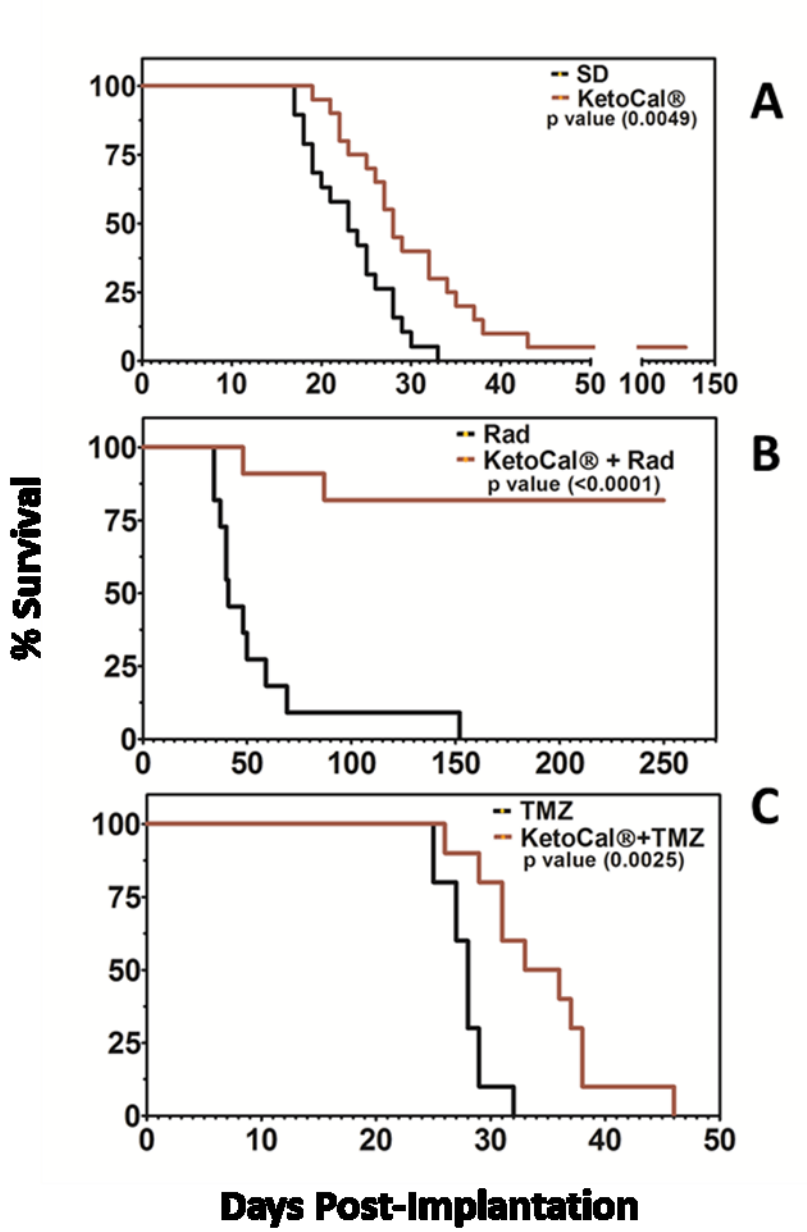


Figure 2: The KD enhances survival of glioma bearing mice. Kaplan-Meier survival plot of animals implanted intracranially with GL261-luc2 malignant glioma cells and (A) maintained on KetoCal [KC, a 4:1 fat:carbohydrate plus protein formulation of the ketogenic diet (KD)] vs. standard diet (SD); (B) treated with 2x4Gy radiation vs.KC plus radiation, and (C) treated with 50 mg/kg temozolomide (TMZ) vs. KC plus TMZ. Animals on KC survived significantly longer when treated with KC alone, when KC was combined with radiation, and when KC was combined with TMZ. [50, 80].

The effectiveness of radiation therapy is due to a number of factors including relative damage done to tumor cells versus normal tissue and the ability of normal cells and tumor cells to repair the damage [81]. Radiation works, in part, by creating ROS through the radiolysis of water. The ROS damage the DNA and other macromolecules, causing sub-lethal damage that can become lethal if not repaired. The potentiation of radiation therapy by the KD or caloric restriction seems paradoxical in light of our data demonstrating a reduction in ROS in tumors from animals maintained on a KD [61]. However, radiation effects do not only occur through ROS, and radiation can directly damage DNA and other cellular macromolecules. Furthermore, in addition to reactive oxygen species, radiation causes the production of reactive nitrogen species (RNS), a potential source of macromolecular damage following radiation [90]. Whether the KD and/or caloric restriction reduces the formation of RNS is as yet unknown. In fact, the main effect of the KD or CR may not be in altering the amount of radiation-induced damage, but may in fact be in modulating the ability of tumor and normal cells to repair radiation-induced damage [91]. Studies have shown that caloric restriction can enhance DNA repair in normal cells [92]; however, this may not be the case in tumor cells, and the differential response of tumor cells and normal cells to genotoxic stress may be mediated by reduced IGF-1 and glucose in the tumor cells. We and others have shown that insulin growth factor is reduced in animals maintained on a ketogenic diet [50, 64, 67, 81]. In addition, a number of studies have shown that reduced activation of the PI3K/Akt pathway, activation of the adenosine monophosphate-activated protein kinase (AMPK) signaling pathway, and reduction of receptor tyrosine kinase growth factor pathways can all reduce radioresistance in tumor cells [93-101].

The variety of effects seen when glucose in lowered and/or ketones are increased suggests that this may also potentiate other therapies, including newer

immune- and targeted therapies. Concerns that potentiation of the anti-tumor effect of a particular therapy may also increase its effect on normal brain are valid; however, we and others have shown that the gene expression changes seen in tumor are different than those seen in normal brain [37, 61, 102]. Further, the KD is known to have neuroprotective effects [29, 74, 103, 104] and thus it has been postulated that this may actually help to protect the normal brain from the deleterious effects of radio and chemotherapy. Taken together, the preclinical data provides strong support for the clinical use of the KD or caloric restriction as an adjuvant therapy for the treatment of gliomas and other cancers.

Chapter 3 of this dissertation will further explore the mechanisms and specifically the role of BHB in the radiation-enhancing properties of the KD.

KD In Human Cancer Patients

Studies of glucose utilization in cancer go back prior to the 1980s, including studies of metabolism and cancer cachexia [40, 105]. These and other studies suggested that the ketogenic diet consisting of a high percentage of medium chain triglycerides (MCT) along with various supplements resulted in weight gain and improved nitrogen balance in both animals and humans. In 1995 Nebeling and colleagues published a case report in which they used a similar ketogenic diet based on MCT oil to treat 2 female pediatric patients with advanced stage malignant brain tumors [106, 107]. They demonstrated that dietary induced ketosis decreased the availability of glucose to the tumor without causing a decrease in patient weight or overall nutritional status. Furthermore, both children had long-term tumor management [107]. The 2nd case report was published in 2010 by Zuccoli and coworkers [108]. This patient was a 65-year-old

female with a multicentric glioblastoma. She was put on a 4:1 (ratio of fats:carbohydrate plus protein) calorie restricted (600kcal/day) ketogenic diet during radiation and chemotherapy. During this time her body weight dropped by 20%, she had reduced blood glucose, increased urinary ketones and, most importantly, no observable brain tumor by either fluorodeoxyglucose Positron Emission Tomography (FDG-PET) or magnetic resonance imaging (MRI). The tumor recurred ten weeks after the patient resumed her normal eating habits and she succumbed to her disease less than 2 years after diagnosis. While this patient did not experience long-term tumor control after cessation of the diet, this report demonstrated that the diet could be tolerated, even when used in a calorie-restricted setting. Results of a phase 1 clinical trial were reported in 2011 by a German group [109]. Tolerability of a restricted calorie ketogenic diet was tested in 16 patients with a variety of advanced (end-stage) cancers. There were no severe side effects and 5 of the 16 patients were able to complete the 3 month treatment. These 5 patients had stable disease while on the diet. Two of the 11 remaining patients died early following the beginning of the trial, one was unable to tolerate the diet and dropped out immediately, 2 patients dropped out for personal reasons, one was unable to continue the diet for more than a month and 3 had disease progression within less than 2 months of starting the diet and one dropped out to resume chemotherapy. While this trial demonstrated tolerability and favorable side effect profile, the antitumor efficacy could not be assessed due to the variety and severity of disease in the patients. Recently, Schwartz et al reported on 2 patients with recurrent GBM treated with a calorie restricted ketogenic diet as a monotherapy and although the diet was tolerated, both patients showed tumor progression - the first within 4 weeks and the second within 12 weeks of beginning the protocol [110]. More recently, a number of prospective clinical trials have been initiated which have been summarized in Table 1.

These trials include studies of up-front treatment using the KD in addition to standard radiation and chemotherapy in patients diagnosed with GBM.

Active Clinical Trials: Ketogenic Diet and Gliomas			
ClinicalTrials.gov Identifier	**Title**	**Phase**	**Location**
NCT01865162	Ketogenic Diet as Adjunctive Treatment in Refractory/Endstage Glioblastoma Multiforme: a Pilot Study (KGDinGBM)	I	MidAtlantic Epilepsy and Sleep Center, Bathesda, Maryland, US
NCT03451799	Ketogenic Diet in Combination With Standard-of-care Radiation and Temozolomide for Patients With Glioblastoma	I	Cedars Sinai Medical Center, Los Angeles, California, United States
NCT01535911	Pilot Study of a Metabolic Nutritional Therapy for the Management of Primary Brain Tumors	I	Michigan State University/Sparrow Hospital, East Lansing, Michigan, United States
NCT02302235	Ketogenic Diet Treatment Adjunctive to Radiation and Chemotherapy in Glioblastoma Multiforme: a Pilot Study (GBMXRT)	II	MidAtlantic Epilepsy and Sleep Center, Bathesda, Maryland, US
NCT02286167	Glioma Modified Atkins-based Diet in Patients With Glioblastoma (GLAD)	I	The Johns Hopkins Hospital, Baltimore, Maryland, US
NCT03278249	Feasibility Study of Modified Atkins Ketogenic Diet in the Treatment of Newly Diagnosed Malignant Glioma	I	UC Health, Cincinnati, Ohio, United States
NCT02939378	Ketogenic Diet Adjunctive to Salvage Chemotherapy for Recurrent Glioblastoma: A Pilot Study	I/II	Beijing Tiantan Hospital, Bejing, Beijing, China
NCT00575146	Ketogenic Diet for Recurrent Glioblastoma	I	Senckenberg Institute of Neurooncology, Frankfurt, Germany
NCT03160599	Restricted Calorie Ketogenic Diet as a Treatment in Glioblastoma Multiforme	I	Guangzhou Medical University, Guangzhou, Guangdong, China
NCT01754350	Calorie-restricted, Ketogenic Diet and Transient Fasting During Reirradiation for Patients With Recurrent Glioblastoma	I	Senckenberg Institute of Neurooncology, Frankfurt, Germany
NCT03075514	Ketogenic Diets as an Adjuvant Therapy in Glioblastoma (KEATING)	I	University of Liverpool, Liverpool, Merseyside, United Kingdom

Table 1: Active clinical trials exploring ketogenic diets in malignant glioma patients.

The case reports described above along with numerous anecdotal reports suggest that the KD may be a promising anti-cancer therapy; however, more work is needed to determine how to best utilize this, and other metabolic therapies for the treatment of tumors. Most of the information regarding the use of the ketogenic diet comes from the epilepsy literature. Further research is needed to determine optimum blood ketone and glucose levels for anticancer effects. In addition, a variety of ketogenic diets are used for seizure control and it is not clear if one or more of the different formulations will provide the best results for cancer patients [111]. Finally, while the KD has a long record of safety in the epilepsy community, side effects that occur when used in combination with cancer therapies may differ in type or severity. This data will come from carefully controlled clinical trials that include input from registered dietitians well-versed in the use of the KD. Patient enrollment into clinical trials requires “buy-in” from the medical community. Physicians must be educated on the therapeutic benefits of metabolic alteration as an adjuvant therapy. As with any decision regarding therapy, the patient’s overall condition, including nutritional status, must be taken into account. As suggested by Klement and Champ [81], cancer patients should be assessed for nutritional needs and tolerability of such interventions.

Concern about patients’ quality of life is sometimes given as a reason not to employ KD. Compliance can be made more difficult by the use of steroids (prescribed for peritumoral edema) that often increase hunger and raise blood glucose levels. To address this, at least one clinical trial (NCT02046187) includes an analysis of both patient and caregiver quality of life. Quality of life measurements are being added to more clinical trials, as the importance of this has become recognized at the national level [112-114]. While some clinicians are concerned compliance will reduce quality of life, the patients that do remain on the KD often comment that this allows them to participate in

their own therapy. Despite these caveats, the existing preclinical data suggesting anti-tumor efficacy and a synergistic effect with standard therapies provides a strong impetus to conduct controlled clinical trials, particularly those that will shed light on the interactions between the KD and other therapies.

Introduction to Immunosuppression and the PD-1/PD-L1 pathway in GBM

Evasion of the immune system has emerged as a crucial hallmark of GBM. These tumors exert a variety of immunosuppressive pressures on the surrounding microenvironment including increased induction of T regulatory cells (Tregs), elevated immunosuppressive cytokine levels, diminished CD4+ helper T cell populations, tolerized antigen presenting cells and upregulated immune inhibitory checkpoints [115]. There are multiple immunotherapeutic approaches for cancer in development [116, 117]; however, the success of therapies targeting immune inhibitory checkpoint pathways in other cancers, particularly those inhibiting the PD-1/PD-L1 axis, have generated increased interest in their use for GBM. Programmed cell death-1 (PD-1) is a receptor found on the surface of activated effector T cells and acts as a checkpoint to regulate immune proliferation and activation. When PD-1 binds its ligand, programmed death ligand 1 (PD-L1), CD8+ and CD4+ T cell activation is suppressed [118]. Tumor-infiltrating lymphocytes can express high levels of PD-1 which may reflect an exhausted phenotype and poor effector function [119]. Increased PD-L1 expression has been observed on tumor cells and immune cells within the GBM microenvironment [119-122] and leads to direct inactivation of CD8+ T cells [123, 124] (Figure 3). Further, many studies have demonstrated that high expression of PD-L1 in tumor tissues is correlated with glioma grade [125].

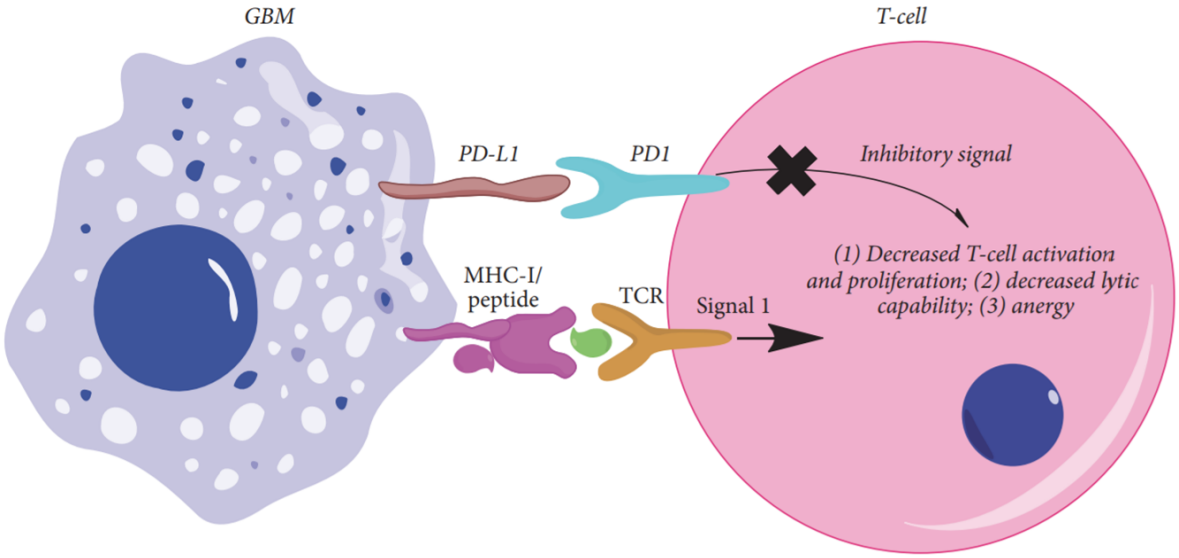


Figure 3: PD-L1 immune inhibitory checkpoint mechanism. Tumor cells can express the inhibitory ligand PD-L1 which binds to the receptor PD-1 on T cells. This lead to decreased activation and effector capabilities of the T cell in the tumor microenvironment [126].

The main focus of our work is to understand and develop the ketogenic diet as an adjuvant to standard therapies and newer emerging therapeutic strategies for brain tumors. We therefore sought to explore the impact of the KD on the anti-glioma immune response and in particular the immune inhibitory checkpoint pathways. We previously published a study demonstrating that the KD alters pathways involved in hypoxia, angiogenesis, invasive potential in a mouse model of malignant glioma [62]. This suggested that the KD may alter the way a tumor shapes and interacts with its microenvironment, leading us to hypothesize that it may also impact specific components of the immune system. These earlier studies taken together with the growing interest in implementing immunotherapeutic strategies for GBM provided the initial impetus for conceiving the studies described in this dissertation.

Chapter Organization

Chapter 2 of this dissertation, titled *Enhanced Immunity in a Mouse Model of Malignant Glioma is Mediated by a Therapeutic Ketogenic Diet*, will discuss the evidence demonstrating that a ketogenic diet works as an immune adjuvant *in vivo*. This work has been published [127] and use in this document is permitted under the Creative Commons Attribution 4.0 International License. This study was completed in collaboration with Danielle Lussier, PhD, who co-authored the publication. This chapter includes the manuscript in its entirety.

Chapter 4 of this dissertation *The Ketone Body Beta Hydroxybutyrate Reduces Expression of PD-L1 on Glioma Cells but Does Not Impact Expression of PD-1 on T cells In Vitro,* will discuss the evidence exploring the ability of the ketone body BHB to recapitulate the effects of the full KD. Namely, this chapter focuses on the impact of BHB

on PD-L1 expression in glioma cells and PD-1 expression on T cells. Potential mechanisms regulating PD-L1 expression including constitutive oncogenic signaling and adaptive expression will be discussed. This work has not yet been published.

Chapter 5 of this dissertation titled, *The Ketogenic Diet Increases the Expression of MicroRNAs* Predicted to Target PD-L1, will discuss data demonstrating that the KD increases the expression of several different microRNAS. These microRNAs target multiple pathways in glioma biology including immune inhibitory checkpoint pathways. This work has not yet been published.

Chapter 6 of this dissertation, titled *Discussion*, will discuss the broader implication of the previous three chapters in improving our understanding of ketogenic diets for the adjuvant treatment of malignant glioma.

CHAPTER 2

ENHANCED IMMUNITY IN A MOUSE MODEL OF MALIGNANT GLIOMA IS MEDIATED BY A THERAPEUTIC KETOGENIC DIET

INTRODUCTION

Glioblastoma multiforme (GBM) is a highly aggressive, heterogeneous brain tumor with poor prognosis [128]. Standard of care includes surgical resection followed by radiation and chemotherapy, however median survival is about 15 months with a two-year survival of 30% and a 5-year survival of <5% in adults [129]. Despite breakthroughs in our understanding of the disease, therapeutic options available for GBM have remained largely unchanged over the past three decades. This has led to only marginal increases in overall patient survival and new therapeutic approaches to enhance brain tumor treatment are warranted.

One novel therapeutic approach for GBM involves targeting a phenotypic trait shared by virtually all cancer cells, deregulated metabolism. It has been postulated that metabolic alteration such as that seen with the therapeutic ketogenic diet (KD) may be an effective anti-cancer strategy [79]. The KD is a high fat, low-carbohydrate/adequate protein nutritional therapy used in the treatment of refractory epilepsy [28]. We and others have shown that the KD enhances survival in mouse models of malignant glioma [38, 43, 61, 80]. We also demonstrated that the KD greatly enhanced survival when administered in combination with radiation [80]. Mechanistically, the KD alters a variety of processes that influence the tumor microenvironment including hypoxia, inflammation,

angiogenesis and vascular permeability [61, 62]. However, the effect of a KD on the GBM tumor-reactive immune response has yet to be examined.

We have recently shown that an unrestricted KD decreases expression of the hypoxia marker carbonic anhydrase IX (CAIX) and the key mediator of the hypoxic response hypoxia-inducible factor alpha (HIF-1α) in a mouse model of malignant glioma [62]. Wei et al. demonstrated that hypoxia leads to inhibition of T cell proliferation and effector responses, with induction of CD4+FoxP3+ T regulatory cells in GBM [130]. This study also demonstrated that this immunosuppressive effect could be reversed by inhibiting HIF-1α. As tumor hypoxia is linked to the less favorable Th2 immune response [131], it is possible that by altering the hypoxic response the KD may promote a Th1 type tumor-reactive immune response. Additionally, we previously demonstrated that the KD reduces activation of the pro-inflammatory transcription factor, nuclear factor kappa B (NF-κB) and reduces expression of cyclooxygenase-2 (COX-2) [61, 62], both of which have been implicated in hypoxia-driven immunosuppression [132]. Taken together these studies led us to hypothesize that the KD may alter the tumor microenvironment to alleviate immune suppression and enhance anti-tumor immunity.

In this study we investigated the role that an unrestricted KD plays in alleviating tumor immune suppression in a mouse model of malignant glioma. We studied the direct effects of the KD on total infiltration and function of tumor-reactive T cells and natural killer (NK) cells, as well as the indirect benefits of the KD on alleviation of immune suppression in the tumor microenvironment.

RESULTS

KD enhanced survival is mediated by CD8+ T cells.

Tumor bearing animals maintained on the ketogenic diet (KD) had a greater median survival when compared to animals fed a standard diet (SD) (Figure 4A). In order to effectively evaluate the importance of tumor-reactive CD8+ T cells in slowing tumor progression, CD8+ T cells were depleted from immune competent albino C57BL/6 mice bearing tumors. There was a significant decrease in survival of mice depleted of CD8+ T cells prior to tumor cell inoculation in comparison to wild type mice maintained on SD (Figure 4B). In order to determine the importance of CD8+ T cells in the anti-tumor effects of the KD, CD8+ T cell depleted animals were treated with the KD and survival was measured. Although the KD significantly improved survival in immune intact mice when compared to those maintained on SD, that difference in survival is lost when CD8+ T cells are depleted and mice are treated with the KD in comparison to immune intact mice fed SD (Figure 4C). Furthermore, the KD significantly increased survival in immune intact mice when compared to CD8 depleted mice fed KD (Figure 4D). Analysis of bioluminescence data also shows slower tumor growth in animals treated with KD when compared to SD in both immune competent and CD8 depleted mice (Figure 4E).

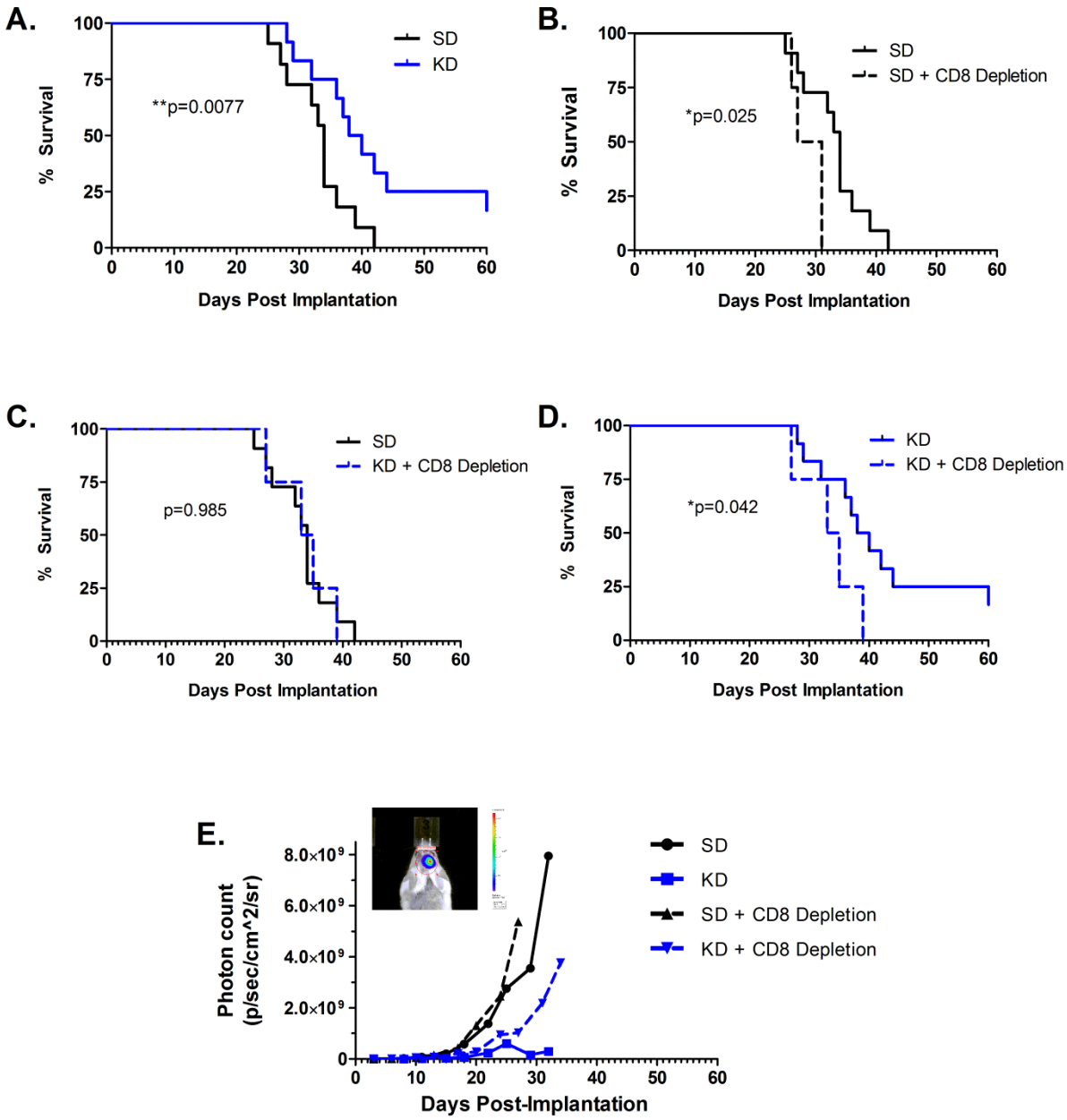


Figure 4: Enhanced survival with the ketogenic diet is mediated in part by CD8 T cells. Kaplan-Meier survival curves for ketogenic diet (KD) versus standard diet (SD) (A), SD versus SD + CD8 depletion (B), SD versus KD + CD8 depletion (C), KD versus KD + CD8 depletion (D). Bioluminescent tumor signals plotted as *in vivo* photon count versus days post-implantation (E). N=12 for immune competent mice; N=5 for CD8 depleted mice; Log-rank (Mantel-Cox) test; p-values indicated on graphs.

The KD enhances immune cell infiltration, and increases the ratio of tumor-reactive CD4+ T cells to Treg ratio.

To evaluate the effects of the KD on immune cell infiltration into the tumor, amounts of tumor-infiltrating CD8+, CD4+, CD4+FoxP3+, and NKp46+CD3- cells were tested. There was no significant difference in the percentage of tumor-infiltrating CD8+ T cells between mice fed the KD and the SD (Figure 5A). However, mice fed the KD had a significant increase in the percentage of CD4+ T cells infiltrating the tumor in comparison to SD (Figure 5B). This increase in the percentage of CD4+ T cells was not due to an increase in the percentage of FoxP3+CD4+ T regulatory (Treg) cells (Figure 4C), and therefore the ratio of CD4+ T cells to Treg cells is significantly increased in tumors from mice fed a KD (Figure 5E). In comparison, the CD8+ T cell to Treg cell ratio remained unchanged when comparing the two treatment groups (Figure 5D). Lastly, there was no difference in the percentage of tumor-infiltrating NK cells in tumors from mice fed a KD compared to SD (Figure 5F). Similar results were found when looking at the total number of infiltrating cells (data not shown). Therefore, the KD enhances CD4+ T cell presence at the tumor site, and this increase is not associated with an increase in the T regulatory cell subset.

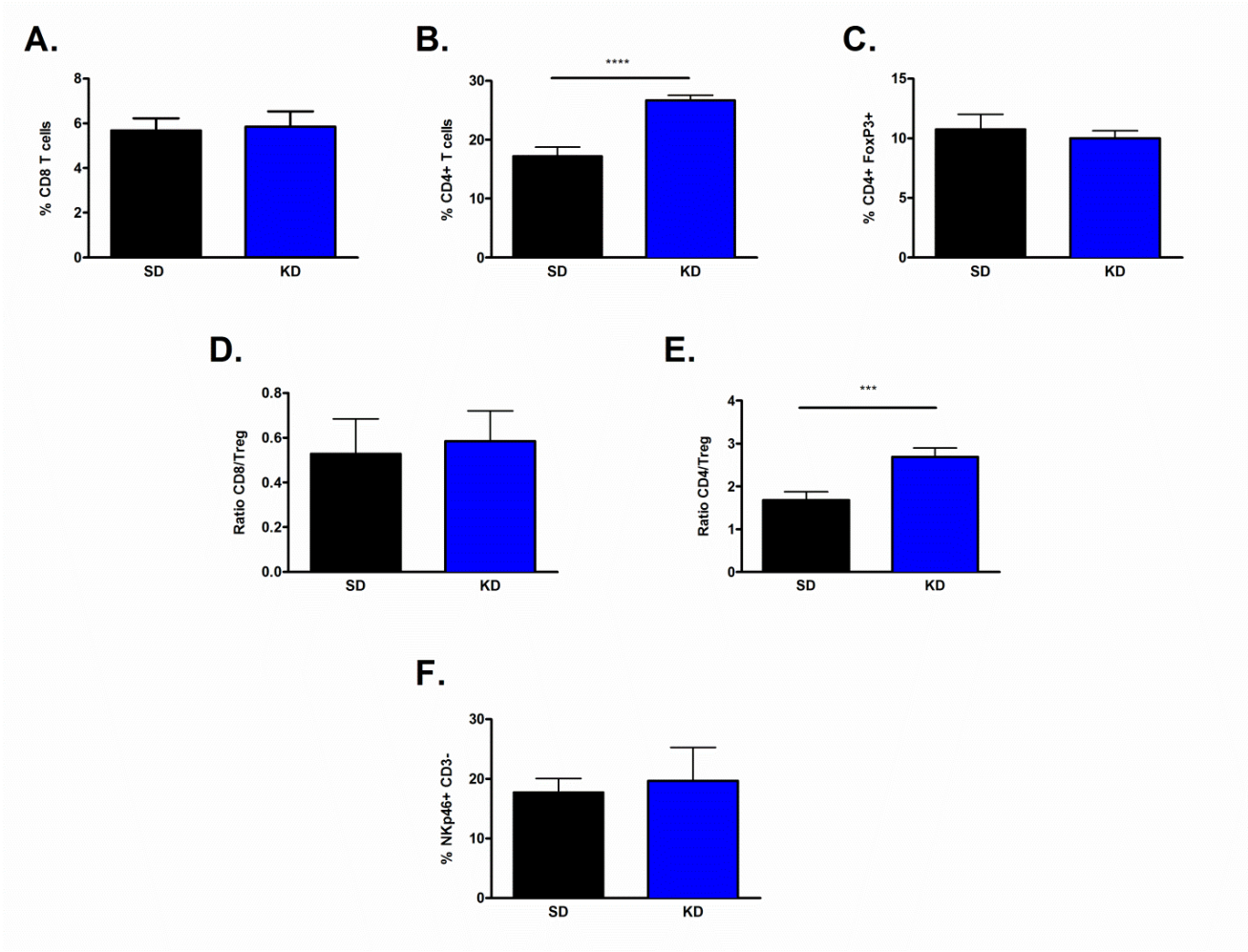


Figure 5: CD4+ T cell infiltration increases in mice fed the KD, without increases in Treg cell numbers. Flow cytometry analysis was performed to assess the cell types infiltrating tumors from mice fed both SD and KD. CD8 T cells (A), CD4 T cells (B) and CD4+FoxP3+ T regulatory cells (C) were assessed. The ratio of CD8 T cells to T regulatory cells (D) and CD4 to T regulatory cells (E) were determined. The percent of infiltrating NKp46+CD3- natural killer cells were also assessed. N=5; student's two-tailed t-test; ***p<0.001; ****p<0.0001.

The KD influences expression of immune inhibitory receptors on tumor-infiltrating lymphocytes, and immune inhibitory ligands on glioma cells.

Tumor cell expression of immune inhibitory checkpoint proteins is a major mechanism by which tumors limit the efficacy of immune responses *in vivo*. To examine the influence of the KD on immune inhibitory checkpoints we evaluated changes of immune inhibitory receptor expression on CD8+ tumor-infiltrating lymphocytes (TILs), and changes in expression of inhibitory ligands on the tumor cells. Mice fed the KD had significantly reduced expression of two inhibitory ligands, PD-1 (Figure 6A) and CTLA-4 on CD8+ TILs (Figure 6B). Additionally, mice fed the KD had reduced expression of CD86 (Figure 6C) and PD-L1 (Figure 6D) on the tumor cells. This suggests that the KD may alter tumor-mediated T cell suppression by reducing the number of cells that are susceptible to inhibition through the PD-1 and CTLA-4 inhibitory pathways.

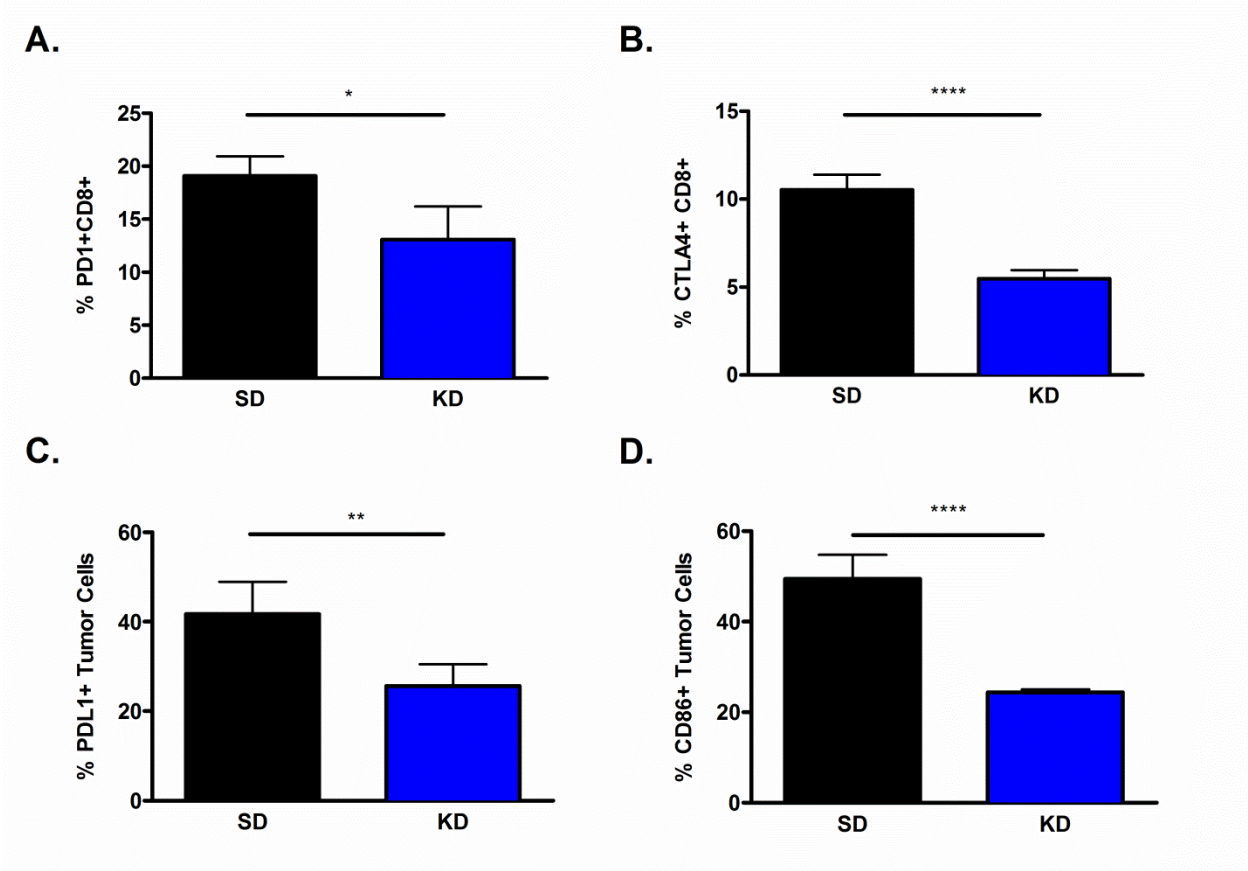


Figure 6: The ketogenic diet reduces expression of immune inhibitory receptors and ligands expressed in glioma tumors. Expression of the immune inhibitory receptors, PD-1 (A) and CTLA-4 (B) on infiltrating CD8 T cells isolated from tumors from mice fed each diet were assessed. Expression of the immune inhibitory ligands, CD86 (C) and PD-L1 (D), on GL261-Luc2 tumor tissue was also assessed. N=5; student's two tailed t-test; *p<0.05; **p<0.01; ****p<0.0001.

The KD enhances innate and adaptive tumor specific immune function against glioma cells.

To evaluate the influence of the KD on tumor-reactive immune cells at the tumor site, immune cell function from TILs removed from the tumor site at time of necropsy was tested. Intracellular cytokine staining following stimulation with tumor cells showed that when compared to SD, the KD significantly increases the ability of tumor-reactive CD8+ T cells to produce interferon gamma (IFN-γ), tumor necrosis factor (TNF), and interleukin 2 (IL-2) when stimulated with GL261-Luc2 cells (Figure 7A). Additionally, the KD significantly increased cytotoxic capabilities of tumor-reactive T cells from mice when compared to SD (Figure 7B). The function of T regulatory cells was also assessed by intracellular cytokine staining for interleukin 10 (IL-10). Although we did not find a difference in the number of tumor-infiltrating Tregs, those found in the tumors from animals fed the KD produced significantly less IL-10 in response to GL261-Luc2 cells when compared to animals maintained on SD (Figure 7C). Lastly, we studied natural killer (NK) cell function and found that tumor-infiltrating NK cells from mice fed the KD produce significantly more IFN-γ and TNF in response to GL261-Luc2 cells than the cells isolated from SD fed animals (Figure 7D). Whether through direct interaction with immune cells, or through alleviation of tumor immune suppression in the microenvironment, the KD significantly enhances tumor-reactive immune function.

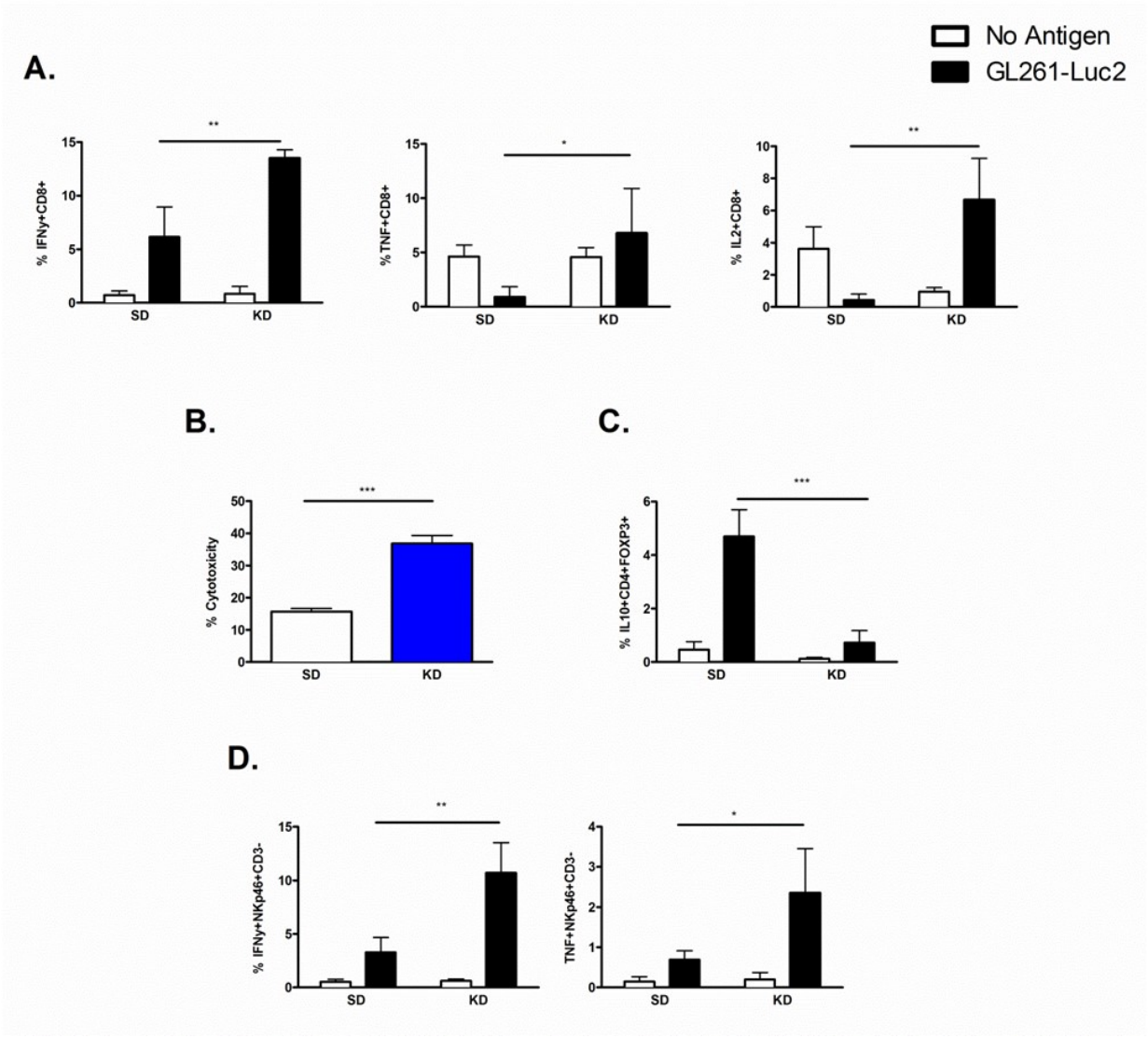


Figure 7: The ketogenic diet significantly enhances tumor-reactive CD8+ T cell and NK cell activity. Tumor-infiltrating lymphocytes (TILs) isolated from gliomas from mice fed KD versus SD were cultured alone (white bar) or in the presence of GL261-Luc2 tumor cells (black bar) to access activity. Analysis of IFN-γ, TNF and IL-2 production in tumor-infiltrating CD8+ T cells was performed (A). Cytotoxic capability of CD8+ T cells isolated from tumors was assessed following exposure to GL261-Luc2 cells (B). IL-10-production in CD4+FoxP3+ T regulatory cells was also assessed in response to stimulation with GL261-Luc2 cells (C). IFN-γ and TNF production in the infiltrating NKp46+CD3- natural killer cells isolated from tumors were assessed (D). N=5; student's two-tailed t-test between the antigen-challenged SD and KD groups only; *p<0.05; **p<0.01; ***p<0.001.

The KD enhances innate and adaptive tumor-reactive immune responses indirectly via alleviation of immune suppression.

To determine if the KD specifically enhances TIL function in the tumor microenvironment or alters global immune status, the effects of the KD on immune responses to two strains of Lymphocytic Choriomeningitis Virus (LCMV) was examined. Non-tumor bearing mice were infected with either LCMV Armstrong or Clone 13, and CD8 T cell function was accessed at Day 6 and 30. There was no significant difference in cytokine production by CD8+ T cells responding to either LCMV dominant epitopes, GP33 or NP396 (Figure 8A) at either time point or with either infection regardless of diet. Additionally, there was no significant difference in the percentage of PD-1+CD8+ T cells between KD and SD fed mice (Figure 8B). Although the KD did not alter CD8 function against acute and chronic viral infections, it did alter immune mediated killing at the tumor site suggesting alleviation of immune suppression is specific to the tumor microenvironment.

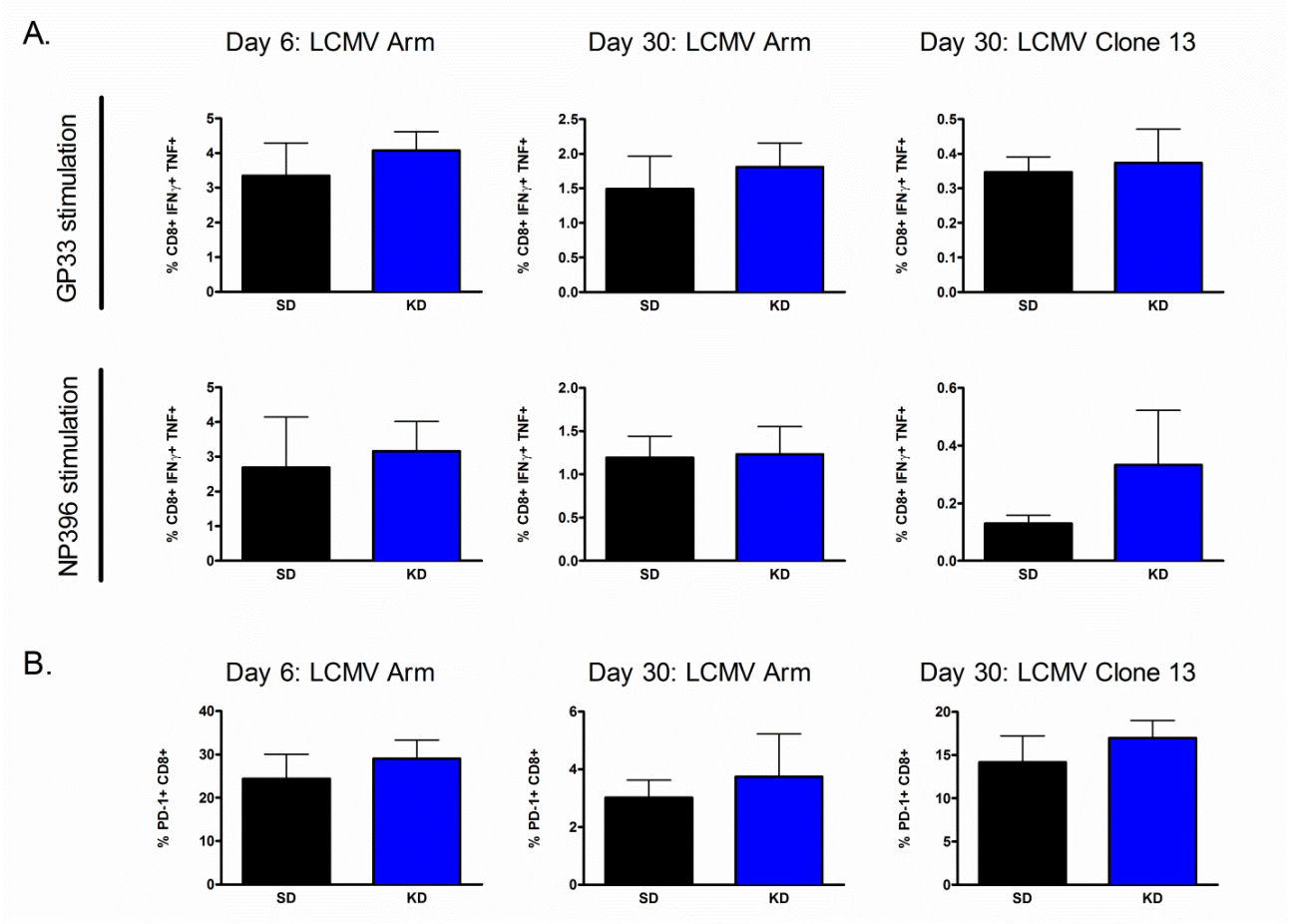


Figure 8: The ketogenic diet had no effect on T cell activity in an acute and chronic mouse model of LCMV infection. Splenocytes from non-tumor bearing mice infected with LCMV Armstrong or Clone 13 were isolated at day 6 and 30, and stimulated with GP33 or NP396 antigens. IFN-γ+TNF+CD8+ cells in mice fed SD versus KD (A). PD-1+CD8+ expression in mice fed SD versus KD (B). N=5 in each group.

DISCUSSION

Activated effector immune responses against glioblastoma multiforme (GBM) may provide benefits in patient survival; however these tumors exert a variety of immunosuppressive pressures on the surrounding microenvironment [133, 134]. These include increased induction of CD8+FOXp3+ regulatory T cells (Tregs), elevated immunosuppressive cytokine levels, diminished CD4+ helper T cell populations, tolerized antigen presenting cells and upregulated immune inhibitory checkpoints [115]. For example, Tregs suppress immune responses by secreting cytokines such as IL-10 and facilitating inactivation of CD8+ cytotoxic T cells by direct cell-to-cell interactions [135]. A key observation in immunosuppressed GBM patients is a decrease in CD4+ T cells with an increased proportion of Tregs and increased IL-10 levels [115, 136]. The current study demonstrated that tumors from animals maintained on the KD had a significantly increased CD4+ T cell population and a decreased proportion of Tregs when compared to control animals. Further the Tregs isolated from animals maintained on the KD produced significantly less IL-10 when stimulated with tumor cells. Similar results were demonstrated in a study using a pancreatic cancer model which showed increased CD4+ T cells and decreased Tregs when animals were fed a KD [137].

In addition to increasing Tregs and IL-10 production in the microenvironment, tumors exploit immune inhibitory signaling pathways involving direct cell-to-cell interactions. Key mediators of this system include cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed death-1 (PD-1) which are found on the surface of activated effector T cells and act as checkpoints to regulate immune proliferation and activation. For example, when PD-1 binds its ligand, programmed death ligand 1 (PD-L1), activated CD8+ and CD4+ T cells are suppressed [118]. Increased PD-L1

expression has been observed on tumor cells and immune cells within the GBM microenvironment [119-122] and leads to direct inactivation of CD8+ T cells [71, 123]. The current study demonstrates significantly decreased expression of PD-1 and CTLA-4 on tumor-infiltrating CD8+ T cells and decreased expression of their ligands (PD-L1 and CD86, respectively) on dissociated tumor cells from animals maintained on the KD when compared to control animals. Blockade of the CTLA-4 and PD1 immune checkpoints represents a potentially important anti-glioma strategy that has proven effective in preclinical models of glioma [138-142] and has warranted exploration in ongoing clinical trials [143].

The current study suggests that the KD may shift the immunological landscape from inflammatory, non-protective immune responses to cytotoxic Th1 responses and promotion of immune mediated killing at the tumor site. Shifting the balance toward a Th1 immune response leads to a general change in cytokine milieu at the tumor site which alters antigen presenting cell maturation and amount of overall immune cell activation [144-149]. This may explain results seen in this manuscript including increased NK and CD8+ T cell function, changes in CD4+ T cell recruitment, reduction in immune inhibitory receptor expression, and ligand availability on the tumor cells themselves. It should be noted that increased CD4 to CD8 T cell ratio may be indicative of a Th2 type immune response at the tumor site [150], which may promote an immune tolerance state; however, greater CD8 T cell activation in the tumors from mice maintained on a KD suggests this is not the case.

It is known that activated T cells undergo metabolic reprogramming in which glycolysis is required to support proliferation and efficient growth [151-154]. Recent evidence also suggests that reduced glucose availability and increased fatty acid

oxidation favors T regulatory cells over effector T cells [155]. However, tumor-infiltrating T cells from mice fed the KD are still able to mount effective responses, undergo appropriate differentiation, and retain function even with the characteristic drop in glucose availability that accompanies the KD. It is currently unclear how the KD alters the metabolic activity of lymphocytes and why this effect appears to be specific to the lymphocytes isolated from the tumor microenvironment. It is possible that T-cells can utilize ketones as a primary energy source in place of glucose in a way similar to that of normal cells in the brain [32, 34]. Recent work has suggested that tumor cells may outcompete other cells in the microenvironment for glucose and other nutrients, thereby reducing the activation of anti-tumor effector T cells [156, 157]. By providing ketones as an alternative energy source for lymphocytes it can be postulated that the KD may alleviate immunosuppression mediated by nutrient competition. Further studies are needed to explore this question and determine the precise role of ketones in T cell metabolism.

While the effect of the KD on tumor-infiltrating lymphocytes has only recently been explored, existing preclinical *in vitro* and *in vivo* data as well as case reports and anecdotal information have generated increased support for clinical testing. This is supported strongly by our preclinical data demonstrating that the KD, when given in combination with radiation, dramatically enhances survival when compared to radiation treatment alone [80]. The mechanisms underlying this effect are still under investigation; however, as radiation-induced tumor killing is known to expose the immune system to a greater diversity of tumor antigens, increased antigen processing, and increased immunogenic cytotoxicity it is possible that the KD as an adjuvant can work to augment the effect of radiation in part by enhancing immunity against GBM.

In summary, the KD may work as an immune adjuvant in the glioma microenvironment by reducing immune suppression, and promoting Th1 type immune responses against the tumor. These data provide additional support for the use of the KD in combination with the current standard of care and newer therapies for the treatment of brain tumors.

METHODS

Antibodies and cell lines

Fluorochrome-conjugated anti-mouse monoclonal antibodies (Abs) specific for CD8α, CD274, CD279, CTLA-4, CD86, tumor necrosis factor (TNF), interferon gamma (IFN-γ), interleukin-2 (IL-2), CD4, FoxP3, NKp46, CD3, and interleukin-10 (IL-10) were purchased from eBiosciences (San Diego, CA) and diluted 1:200 prior to use. Anti-CD8 depletion antibodies were purified from the mouse 2.43 hybridoma cell line purchased from ATCC (Manassas, VA). Bioluminescent GL261-Luc 2 cells were derived and grown as previously described [80].

Mice and tumor implantation

GL261-Luc2 cells were harvested by trypsinization, washed and resuspended at a concentration of 1–2x107 cells/ml in DMEM without FCS and implanted into ten week old B6 (Cg)-Tyrc-23/J (albino C57BL/6) mice (The Jackson Laboratory, Bar Harbor, ME) at an average weight of 19-20 grams as previously described [61, 80, 158]. Briefly, animals were anesthetized by an intraperitoneal injection of ketamine (10 mg/kg) and xylazine (80 mg/kg), placed in a stereotactic apparatus and an incision was made over the cranial midline. A burrhole was made 0.1 mm posterior to the bregma and 2.3 mm to

the right of the midline. A needle was inserted to a depth of 3 mm and withdrawn 0.4 mm to a depth of 2.6 mm. Two µl of GL261-luc2 cells (10^7 cells/ml) were infused over the course of 3 minutes. The burrhole was closed with bonewax and the incision was sutured.

Treatment and Animal Monitoring

Following implantation surgery, animals were fed standard rodent chow for 3 days. Animals were then randomized to remain on standard diet (SD) or changed to a KD (KetoCal®; Nutricia North America, Gaithersburg, MD). The KD was obtained directly from the manufacturer and is a nutritionally complete diet providing a 4:1 ratio of fats to carbohydrates plus protein (72% fat, 15% protein, and 3% carbohydrate). The KD was prepared by mixing KetoCal® with water (2:1) and fed to the animals each day (*ad libitum*). Bioluminescence was analyzed to quantify tumor burden as described [80]. Serum β-hydroxybutyrate (BHB) and glucose levels were measured using a Precision Xtra® blood monitoring system (Abbott Laboratories, Abbott Park, IL). Animals were weighed every 3-5 days and euthanized upon occurrence of visible symptoms of impending death such as hunched posture, reduced mobility and weight loss [61, 159]. Measurements of animal body weight, blood BHB, and glucose were also performed (data not included).

CD8 depletion in vivo

Supernatant from 2.43 hybridoma cells was precipitated in saturated ammonium sulfate to 45% (v/v) overnight at 4°C and dialyzed against PBS for 24 hrs. The concentration of dialyzed antibody was determined by UV spectroscopy, and 0.3 mg of purified antibody was administered via intraperitoneal injection twice before tumor inoculation (day -5 and -3), and continued every three days after inoculation until

euthanasia. CD8 T cell depletion was confirmed by flow cytometry analysis of peripheral blood mononuclear cells, as previously described [160].

Tissue preparation

When mice became symptomatic they were anesthetized with 80mg/kg ketamine, 10 mg/kg xylazine followed by cardiac perfusion with ice-cold RPMI media just prior to euthanization. Tumor tissue and non-tumor contralateral brain were collected in RPMI media and run through a 70μm filter. Tumor-infiltrating cells were isolated from tumor tissue by centrifugation over a 30/70% Percoll gradient (Sigma-Aldrich, St. Louis, MO) before antibody staining and analysis of cell populations on an LSRFortessa flow cytometer (BD Biosciences, San Jose, CA). Flow cytometry data were analyzed with FlowJo8.8 (Tree Star Inc., Ashland, OR) and graphs were generated using Prism 5 software (GraphPad Software, La Jolla, CA).

Intracellular cytokine staining

Lymphocytes were cultured alone or stimulated with GL261-Luc2 cells at a density of 10^6 cells per well (6-well plate). GolgiStop (BD Biosciences) was added at 1 hour to inhibit export of cytokines and after a further 5 hours of incubation, cells were stained for extracellular proteins. Permeabilization and intracellular staining for cytokines was done according to manufacturer's instructions using the Cytofix/Cytoperm kit (BD Biosciences).

Cytotoxicity ELISA

Lymphocytes were isolated from tumor tissue, and cultured alone or with GL261-Luc2 cells at varying effector to target cell ratios. Lactate dehydrogenase (LDH) ELISA

was performed using CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). Absorbance was recorded at 490nm.

Animals and virus

Six to 8-week-old female C57BL/6 mice were obtained from The Jackson Laboratory. All experiments were conducted under Arizona State University IACUC approval and followed all relevant federal guidelines and institutional policies. The Armstrong and clone 13 strains of Lymphocytic Choriomeningitis Virus (LCMV) were grown as previously described [161]. Mice were infected with 2×10^5 PFU of LCMV (Armstrong) injected intraperitoneally or 2×10^6 PFU of LCMV (clone 13) injected intravenously.

Statistical Methods

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). All values are represented as the mean ± SD and significance was determined using both the Student's t- test and the Mann Whitney non-parametric test. $P < 0.05$ was considered statistically significant. For the Kaplan Meier survival data the log-rank (Mantel-Cox) test was used to assess statistical significance.

CHAPTER 3

THE KETONE BODY BETA-HYDROXYBUTYRATE INHIBITS HISTONE DEACETYLASES AND SENSITIZES MALIGNAT GLIOMNA CELLS TO RADIATION

INTRODUCTION

Despite decades of innovative research, glioblastoma multiforme (GBM) remains a uniformly lethal disease. Radiotherapy is the most effective non-surgical therapy for GBM; however these tumors often recur, lending to the dismal prognosis for this disease. Resistance to radiation and is considered a major driver of recurrence in GBM and other cancers and thus has sparked great interest in the development of new therapeutic strategies that enhance radiation.

An adjuvant strategy for malignant glioma being developed in our laboratory involves using a therapeutic ketogenic diet (KD). Our previously published studies demonstrate enhanced survival and alterations in major pathways involved in glioma growth and progression when glioma-bearing mice are fed an unrestricted KD and [61, 62, 80, 127]. Other groups have demonstrated anti-proliferative effects using alternative methods to increase ketones and reduce glucose including caloric restriction and/or restricted ketogenic diets in experimental mouse and human brain tumor models [79]. While these studies provide important insight into the mechanisms underlying the anti-tumor effects of the KD alone, clinically this treatment is not likely to be given without standard of care consisting of radiation and temozolomide chemotherapy. We therefore investigated the impact of the KD on radiation *in vivo*. Using a bioluminescent, syngeneic intracranial model of malignant glioma (GL261-Luc2), the KD was shown to significantly potentiate the anti-tumor effect of radiotherapy [80]. In this study, 9 out of 11 animals

maintained on the KD and treated with radiation had complete and sustained remission of their implanted tumors, even after being switched back to a standard rodent diet (Figure 2). To our knowledge this is the only study investigating the effects of the KD as an adjuvant to radiation in glioma, warranting further exploration into the radiosensitizing mechanisms of the KD.

It has recently been shown that β-hydroxybutyrate (BHB) can act as a histone deacetylase inhibitor (HDACi) [53]. The epigenetic changes that occur with inhibition of HDACs are being looked at for cancer therapy; and HDAC inhibition can sensitize tumor cells to radiation and chemotherapy [162-165]. These studies along with the finding that BHB acts as an HDAC inhibitor, suggest that this may be an important, but overlooked, mechanism through which the ketogenic diet exerts some of its anticancer effects and enhances radiation. Further, accumulating experimental evidence shows that radiation induces immunogenic cell death and promotes recruitment and function of T cells within the tumor microenvironment [166-168]. So understanding the radiosensitizing mechanisms underlying the KD may also help us understand its role as an immune adjuvant.

In this study we investigated the role BHB plays in radiosensitization. We examined the ability of BHB to inhibit HDACs and radiosensitize malignant glioma cells to radiation *in vitro*. We also examined the impact of BHB on DNA damage repair in these cells. The overall goal of this study is to define the role BHB plays in radiosensitivity with the aim of better understanding the mechanisms underlying the KD as an adjuvant to radiation therapy.

RESULTS

BHB sensitizes mouse malignant glioma cells to radiation

To assess the impact of BHB on radiation sensitivity in vitro, GL261-Luc2 cells were treated with 2, 5 and 10mM BHB with or without one dose of 2 Gy radiation. GL261-Luc2 cells treated with 5 and 10mM BHB alone showed significantly reduced proliferation while treatment with 2mM BHB alone showed no significant changed compared to mock (Figure 9). However, all three doses of BHB when combined with 2 Gy radiation significantly reduced proliferation compared to radiation treatment alone (Figure 9).

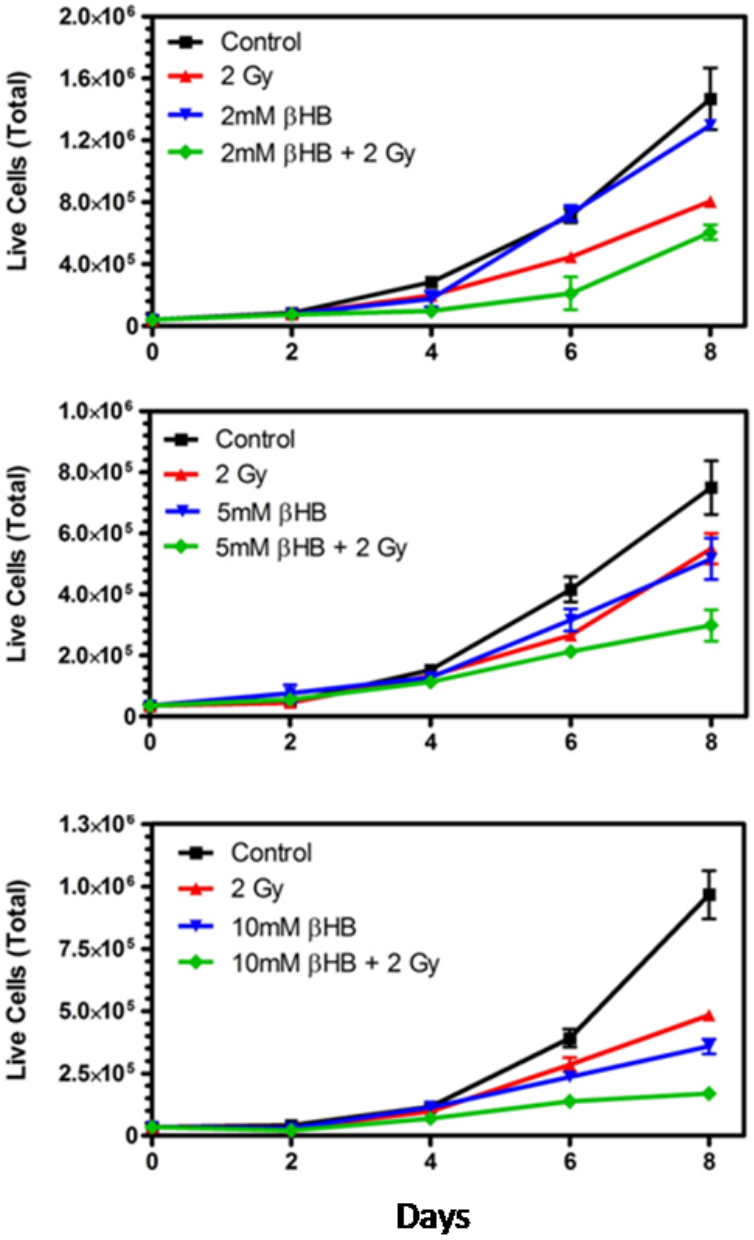


Figure 9: BHB enhances radiation against glioma cells *in vitro*. GL261-Luc2 cells were treated with 2, 5 and 10 mM BHB for 48 hours prior to treatment with 2 Gy radiation. Cells were counted every 48 hours and BHB was replenished every 24 hours throughout the experiment (N=3).

BHB treatment leads to increased accumulation of DNA damage

To determine the impact of BHB on DNA damage, the expression of the double-stranded DNA break marker, γ-H2AX, was assessed. GL261-Luc2 treated with 5mM BHB showed a significantly higher level of γ-H2AX expression at 1 hour post radiation (Figure 10). Interestingly BHB without radiation causes a slight increase in DNA damage although this was not statistically significant. This is a phenomenon observed in other studies and may be related to the genomic instability of cancer cells and the ability of HDACi to slow cell cycle progression [169-171].

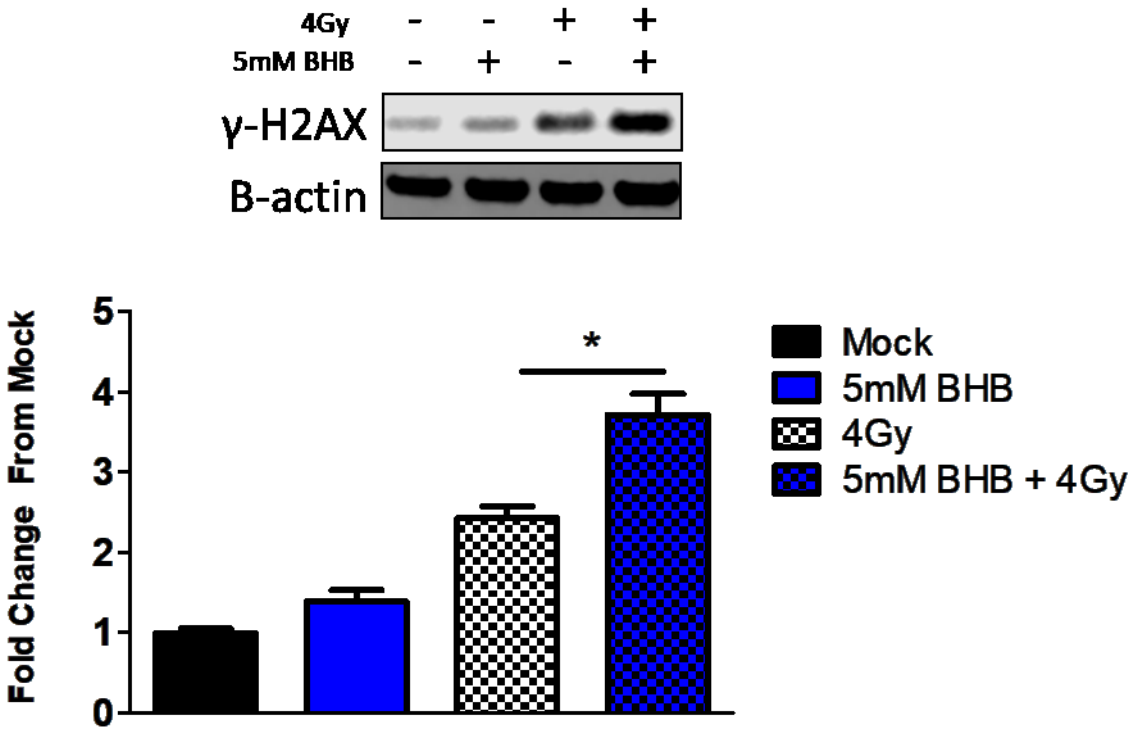


Figure 10: BHB increases radiation-induced DNA damage *in vitro*. GL261-Luc2 cells were treated with 5 mM BHB for 24 hours prior to treatment with 4 Gy radiation. Protein was isolated 1 hour following radiation and western blotting was used to assess levels of the DNA double stranded break marker γ-H2AX. Expression is normalized to B-actin and expressed as a fold change from mock treated cells (N=3; t-test; *$p<0.05$).

BHB significantly increases acetylation of histone H3

To validate the ability of BHB to inhibit HDACs, acetylation of histone H3 was assessed in BHB-treated GL261-Luc2 cells. Treatment with 5mM BHB alone lead to the significant increase in acetylated histone H3 when compared to mock treated cells (Figure 11). Radiation alone increased acetylation compared to mock treated cells. Further, 5mM BHB combined with 4 Gy radiation led to significantly increased acetylation compared to radiation alone (Figure 11).

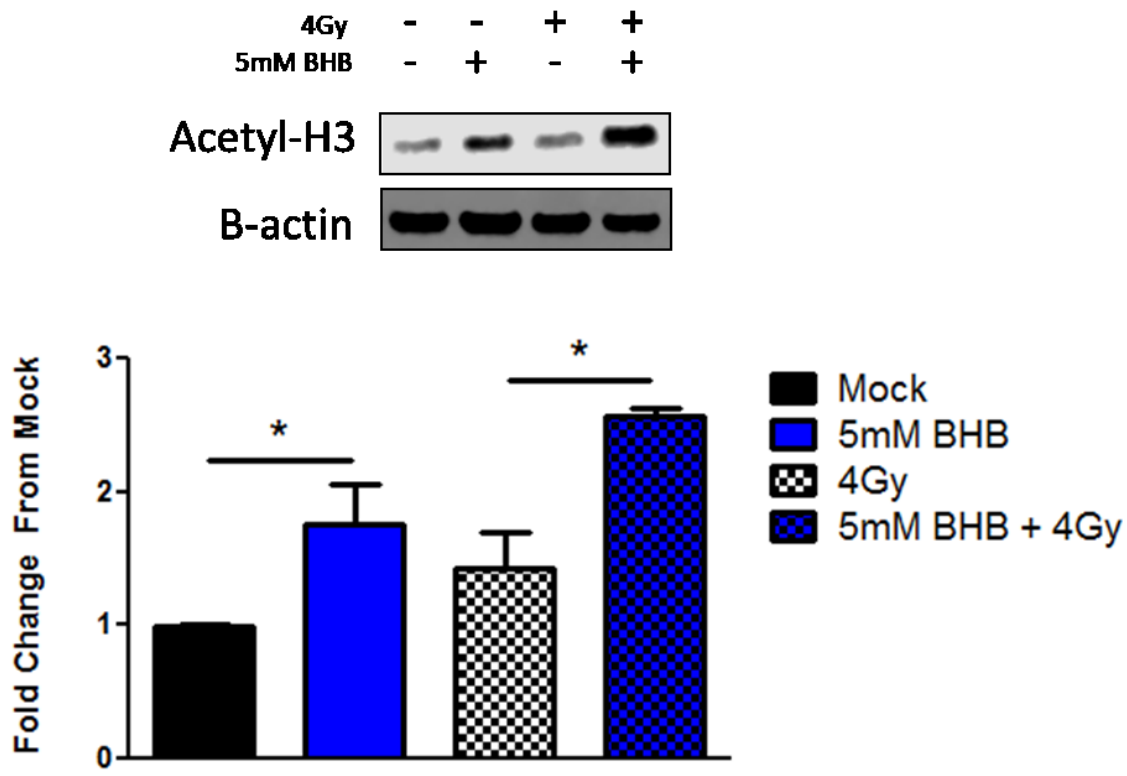


Figure 11: BHB increases acetylation of histone H3 *in vitro*. GL261-Luc2 cells were treated with 5 mM BHB for 24 hours prior to treatment with 4 Gy radiation. Protein was isolated 1 hour following radiation and western blotting was used to assess levels of acetylated histone H3. Expression is normalized to B-actin and expressed as a fold change from mock treated cells (N=3; t-test; *p<0.05).

BHB treatment leads to an increase of cells in the G2 phase of the cell cycle

The different phases of the cell cycle can greatly impact radiosensitivity [172]. To determine the impact of BHB on the cell cycle progression, cell cycle analysis was performed on GL261-Luc2 cells following treatment with 5mM BHB and 4 Gy radiation. BHB alone reduced the percentage of cells in the G1 and increased the percentage of cells in G2 phase, which is considered the most radiosensitive phase (Figure 12). There were similar trends found in the cells treated with both 5mM BHB and 4 Gy radiation; however, only the difference in G1 phase cells was statistically significant when compared to radiation treatment alone.

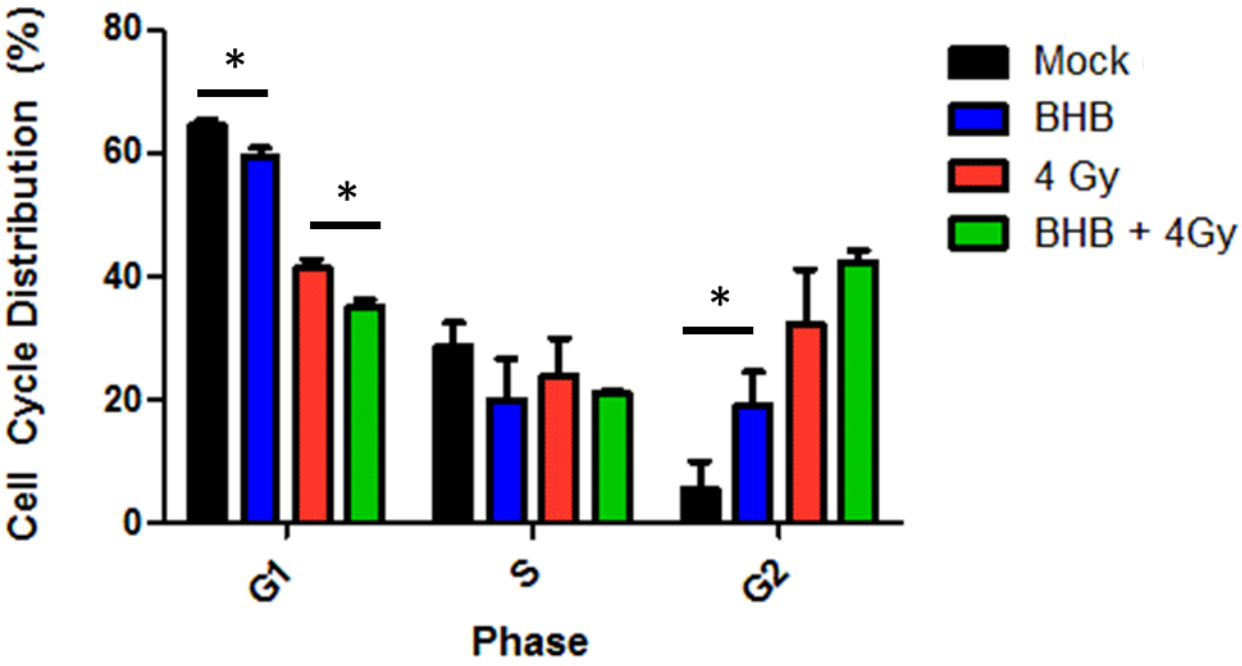


Figure 12: BHB increases G2 arrest in glioma cells *in vitro*. GL261-Luc2 cells were treated for 48 hours with 5mM BHB prior to treatment with 4 Gy radiation. At 24 hours post-radiation cells were fixed and stained with DAPI. Flow cytometry was performed to determine the cell cycle distribution (N=3; t-test; *p<0.05).

BHB reduces the expression of RAD51 and other DNA damage repair proteins in a dose-dependent manner

Many studies have demonstrated that HDAC inhibition can alter DNA damage repair pathways in cancer cells [171]. To determine the impact of BHB on DNA damage repair pathways, the expression of various proteins involved in DNA damage repair was assessed. BHB treatment significantly decreased the expression of MRE11, KU70 and RAD51 when compared to mock treated cells (Figure 13). This corresponded with a dose-dependent increase in acetylation of histone H3.

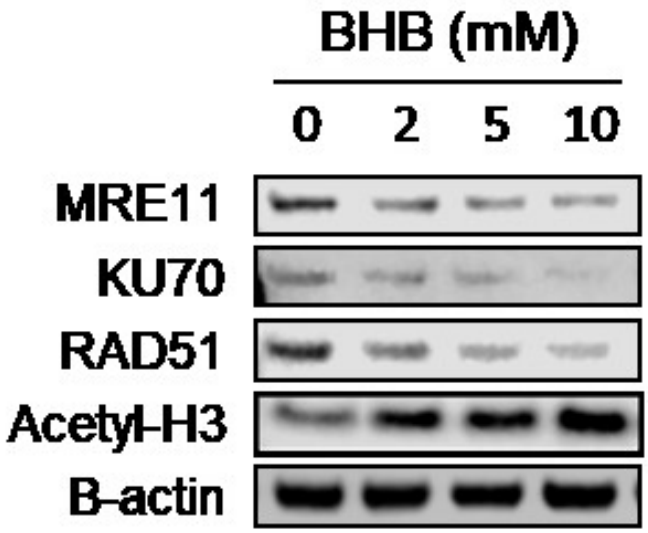


Figure 13: BHB reduces expression of DNA damage repair proteins. GL261-Luc2 cells were treated with 5 mM BHB for 24 hours prior to protein isolation and western blotting analysis was performed using antibodies against MRE11, KU70, RAD51 and acetyl-histone H3. B-actin was used a loading control (experiment repeated in duplicate).

Knockdown of HDAC1, HDAC2 and HDAC3 lead to a decrease in RAD51 expression

BHB is a known class I HDAC inhibitor [53]. To determine the impact of class I HDACs on RAD51 expression, cells were transfected with siRNAs targeting HDACs 1-3. Knockdown of HDAC1, HDAC2, and HDAC3 each led to the significantly reduced RAD51 protein expression in GL261-Luc2 cells (Figure 14A). Knockdowns were confirmed using western blot analysis (Figure 14B).

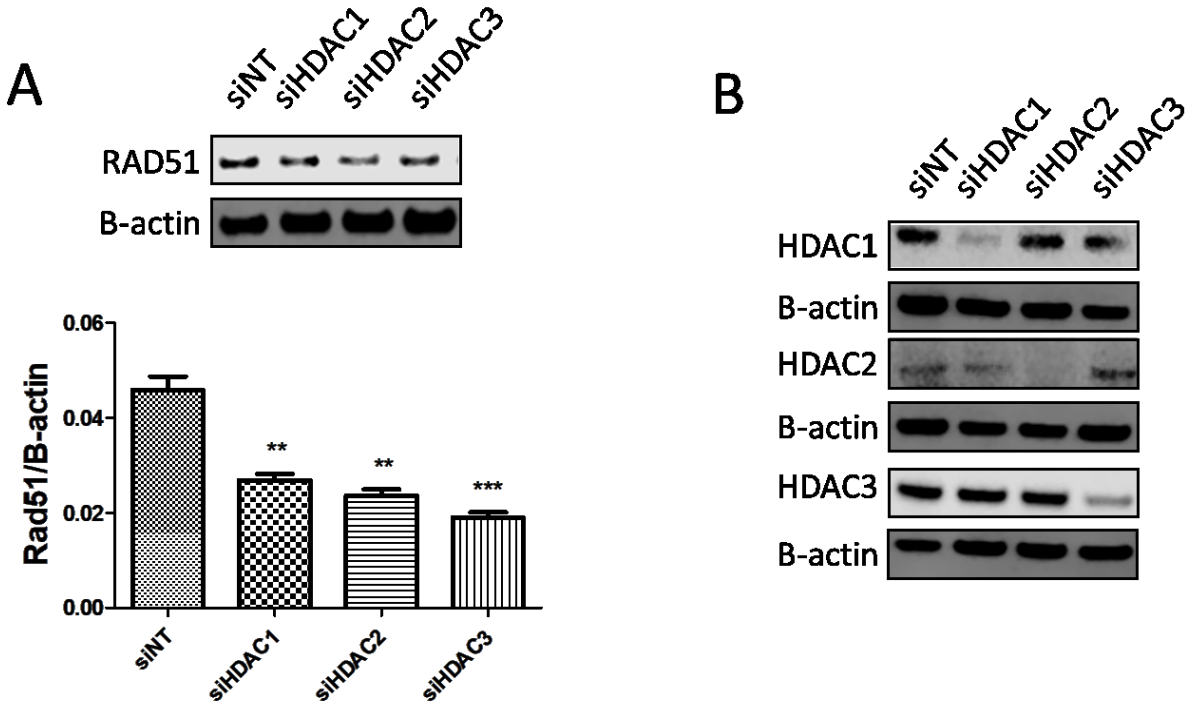


Figure 14: Knockdown of Class I HDACs downregulated expression of RAD51. GL261-Luc2 cells were transfected with siRNAs targeting Hdac1, Hdac2, and Hdac3 or non-targeting siRNAs (siNT) as a control for 72 hours prior to protein isolation. (A) Western blotting analysis was performed using an antibody against RAD51 and expression was normalized to B-actin (N=3; t-test; **p<0.01; ***p<0.001). (B) Knockdowns were confirmed using western blot analysis.

The ketogenic diet reduces expression of RAD51 in vivo

Evidence suggests that several changes elicited by a ketogenic diet (KD) may be due in part to the action of BHB. To determine if the BHB-induced changes in RAD51 expression *in vitro* are also found *in vivo*, expression in tumor tissue from animals maintained on the KD was assessed. Tumor tissue from GL261-Luc2 tumor-bearing mice maintained on the KD shows a significantly decreased expression of RAD51 when compared to mice fed a standard rodent diet (SD)(Figure 15).

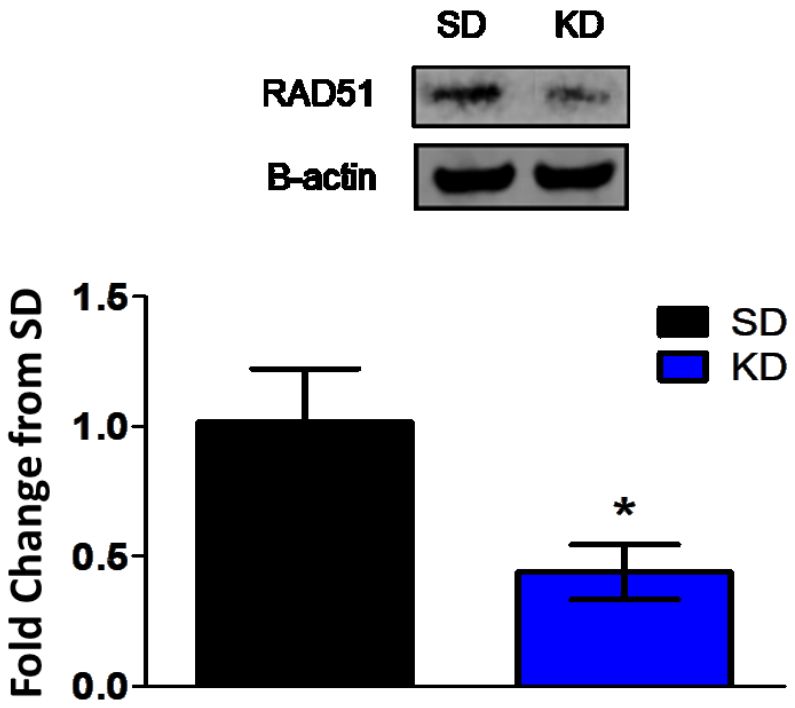


Figure 15: RAD51 expression is reduced in tumors from animals maintained on the KD. Western blotting analysis was performed to assess RAD51 expression on tumor tissue from animals maintained on the ketogenic diet (KD) and compared to that from animals maintained on a standard rodent diet. Expression was normalized to B-actin and displayed as a fold change from SD (N=5 for KD; N=6 for SD; t-test; *$p<0.05$).

DISCUSSION

Dysregulation of epigenetic modifications is a hallmark of many cancers including high grade gliomas [173]. Thus exploring agents that reverse these aberrant epigenetic changes has become a promising anti-cancer strategy [162, 174-176]. It has been postulated that the ketogenic diet (KD) may impact the epigenetic landscape due in part to the activity of the ketone body BHB to inhibit. Shimazu *et al* was the first to demonstrate that BHB inhibits activity of class I histone deacetylases (HDACs) leading to increased histone acetylation and gene expression changes in normal untransformed kidney cells [177]; however until now this has yet to be explored in the context of glioma. Several cancers including GBM, frequently overexpress HDACs, HDAC inhibition has can lead to cell cycle arrest, promote cellular differentiation and induce apoptosis [174, 178]. Although HDAC inhibitors can impact several different pathways in gliomas [178], it has been demonstrated that that Class I HDAC inhibitors can impair DNA damage repair [171]. The current study is the first to validate the HDAC-inhibiting activity of BHB in glioma cells and demonstrate its ability to impact DNA damage repair.

DNA damage repair mechanisms play a key role in the radioresistance of GBM [179]. For example, RAD51 is crucial for homologous recombination repair of double-stranded DNA breaks and is overexpressed in several human cancers including GBM [180, 181]. The increased expression of RAD51 in cancer leads to resistance to double-stranded break inducing anti-cancer therapies [182-184]. A variety of studies have demonstrated that Rad51 inhibition is in effective radiosensitizing strategy for malignant glioma [185-188]. The current study found that BHB significantly reduced the expression of proteins involved in DNA damage repair including RAD51. Further, recent studies have highlighted RAD51 as an effective target for glioma stem cells as well [189-191],

which represent a therapy-resistant population in GBM tumors [192]. We also demonstrated that BHB increases radiosensitivity and decreases expression of RAD51 in human glioma stem cells [193]. Thus BHB-mediated downregulation of RAD51 may represent an important radiosensitizing mechanism underlying ketogenic therapy and may help explain our previously published in vivo results [80].

The ability of HDAC inhibitors to impact expression of proteins involved with DNA damage repair is well supported in the literature. Various HDAC inhibitors have been demonstrated to down-regulate Rad51 in a variety of cancers including pancreatic [194], melanoma [195], acute myeloid leukemia [196], esophageal [197], and colon [198]. However, many of these pharmacological agents have shown some toxicity and limited efficacy making translation into the clinical setting a challenge [171, 174, 199-202]. The current study shows that tumors in glioma-bearing mice maintained on a ketogenic diet (KD) have reduced expression of RAD51. To our knowledge this study is the first to show therapeutically relevant changes that occur due to a non-pharmacologic intervention such as the KD.

HDAC inhibitors are not only being explored for their radiosensitizing capabilities but studies also suggest that they may modulate the immune system. For example, one study found that that the HDAC inhibitor trichostatin A improved the immune response in a glioma model by enhancing the function of natural killer (NK) cells [203]. In *Chapter 2*, we demonstrated that NK cells isolated from tumors maintained on the KD showed increased production of IFN-γ and TNF suggesting increased function. There are now multiple studies in a variety of cancers suggesting that HDAC inhibition is immunomodulatory and may enhance the efficacy of immunotherapy including PD-1/PD-L1 blockade [204-208]. This suggests that the HDAC inhibiting properties of BHB may also enhance the immune system and may shed light on the immune enhancing

properties of the KD introduced in *Chapter 2.* Future studies specifically connecting these epigenetic mechanisms with the immune system and immunotherapy in the context of the KD are warranted.

The results in this study lend to the growing body of evidence suggesting that in the absence of glucose restriction, BHB has anti-tumor benefits on its own. In addition, it provides a potential mechanism underlying the potent radiosensitizing capabilities of the KD demonstrated in our previously published preclinical study. This becomes crucial as more clinical trials are exploring the KD as an upfront therapy with radiation and chemotherapy. Finally, as radiation is known to act in synergy with the anti-tumor immune response and may enhance immunotherapies [166, 168], the current study also suggests that KD and BHB may further enhance the anti-tumor immune response by augmenting the effect of radiation. Taken together these data provide further support for the use of the KD as a pluripotent adjuvant treatment for malignant glioma and may help inform its clinical use.

METHODS

Cell Culture

GL261-Luc2 cells (mouse malignant glioma) are grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). Cells are incubated at 37°C and 5% CO_2.

Treatments

(R)-(-)-3-Hydroxybutyric acid, sodium salt (BHB; Sigma-Aldrich, St. Louis, Missouri) was dissolved in media and replenished every 24 hours for growth assays. Radiation was administered with an RS 2000 Biological Irradiator (Rad Source Technologies Inc., Suwanee, GA) at 2.287 Gy/Min for varying times to attain desired dose.

Growth Assays

GL261-Luc2 murine glioma cells were seeded at 3.5 X 10^4 cells/well in 12-well culture plates with 5mM BHB. The cultures were seeded with BHB 24 hours prior to irradiation. Cell counts were obtained every 48 hours for 192 hours. To count each well, the cells were trypsinized, combined with Trypan Blue (Life Technologies, Carlsbad, CA) and counted using a Countess® automated cell counter (Life Technologies).

Western Blotting

Protein was extracted from cells using RIPA buffer (Cell Signaling Technology). Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). All primary antibodies used were from Cell Signaling Technology and were diluted 1:1000 in blocking buffer prior to use. Secondary antibodies were purchased from Licor Biosciences and were used at a dilution of

1:15,000. Images were obtained using a Licor Odyssey Fc imager and data was analyzed using the Licor Image Studio software.

Cell Cycle Analysis

GL261-Luc2 cells were seeded in a 6-well culture plate at 1.5 x 10^4 cells/mL. Cells were treated with 5 mM BHB for 48 hours prior to treatment with 4 Gy radiation. Twenty-four hours post-radiation cells were lifted with Accutase (Life Technologies, Carlsbad, CA), fixed in 70% EtOH, stained with 1.25µg/mL DAPI (BD Pharmingen, San Jose, CA), and analyzed by FlowJo v10 software.

siRNA Knockdown Experiment

GL261-Luc2 cells were transfected with 25 nmol mouse Hdac1, Hdac2, Hdac3, or non-targeting control siRNAs using using DharmaFECT 1 transfection reagent (all reagents from GE Dharmacon, Lafayette, CO). After 72 hours of transfection protein was isolated and western blotting was performed as described above using antibodies against RAD51. Western blotting was used to confirm the knockdown of HDACs. Transfection conditions were optimized in GL261-Luc2 cells using lamin A/C control siRNAs from Dharmacon.

Tumor Tissue for Western Blotting

Mice were implanted with GL261-Luc2 cells and maintained on the KD or standard rodent diet as previously described in *Chapter 2*. On day 25 post-implantation

tumor tissue was harvested and snap frozen in liquid nitrogen. Tissue was homogenized in RIPA buffer and protein was isolated prior to western blotting analysis for RAD51 expression (western blotting described above).

CHAPTER 4

THE KETONE BODY BETA HYDROXYBUTYRATE REDUCES EXPRESSION OF PD-L1 ON GLIOMA CELLS BUT DOES NOT IMPACT PD-1 EXPRESSION ON T CELLS

INTRODUCTION

The ketone body β-hydroxybutyrate (BHB) has traditionally been thought of as simply a metabolic substrate that replaces glucose during the ketogenic diet (KD), fasting or exercise; however, the effects of increased ketones go beyond simple considerations of energy availability [54, 209]. *In vitro* investigations demonstrated that BHB is able to recapitulate, in part, the *in vivo* effects of the full KD [50, 52]. This suggests that the ketone bodies themselves possess antitumor effects, and that perhaps the effects of the KD are mediated, at least in part, by the ketone bodies. Additional evidence for this comes from data showing that the use of ketone supplementation can enhance the effects of the KD and may even be effective in some diseases when used alone [59, 210-215].

Our laboratory's investigations into the interactions between BHB and glioblastoma cells have revealed insights into the molecular basis for some of the KD's effects, most notably its radio- and chemo-sensitizing effects. *In vitro* studies using BHB demonstrated that, even in the presence of high glucose, physiologically relevant doses of BHB reduced proliferation of several human glioblastoma cell lines, two human cancer stem cell lines, and a murine glioma cell line (unpublished data). In *Chapter 3* of this dissertation we demonstrated the BHB radiosensitizes malignant glioma cells by inhibiting histone deacetylases and reducing expression of DNA damage repair proteins. Further, in a separate study BHB potentiated the chemotherapeutic agent 1,3-bis(2-

chloroethyl)-1 nitrosourea (BCNU, carmustine) in a cell line derived from a recurrent human glioblastoma [50]. Taken together, these data suggest that ketone supplementation may provide an effective, less stringent alternative to the rigors of the KD; yet additional studies are needed to further develop this approach.

It was demonstrated in *Chapter* 2 that the KD works in part as an immune adjuvant, boosting tumor-reactive immune responses in the glioma microenvironment by alleviating immune suppression *in vivo.* In particular we found that KD reduced expression of programmed cell death-1(PD-1) on tumor-infiltrating T cells and its ligand PD-L1 on tumor cells. The PD-1/PD-L1 axis represents a crucial mechanism of glioma-mediated immunosuppression and is a major focus of immunotherapy in GBM and other cancers [125]. The impact of BHB alone on this pathway is not yet understood and warranted the current study.

In this study we found that BHB treatment significantly reduces expression of PD-L1 on tumor cells which is consistent with our *in vivo* results. However; BHB failed to alter expression of PD-1 on T cells culture *in vitro*. These results suggest that BHB may play a key role in enhancement of anti-tumor immunity by KD but may not directly alter immune checkpoints in T-cells outside of the context of a tumor microenvironment. Taken together these results continue to increase our understanding of the mechanisms underlying the KD and specifically the role of BHB.

RESULTS

BHB reduces expression of PD-L1 on malignant glioma cells

To determine if BHB is plays a role in the downregulation of PD-L1 seen *in vivo* with the full KD, GL261-Luc2 mouse glioma cells were treated with BHB *in vitro* prior to analysis of PD-L1 expression. Western blotting analysis shows that 5mM BHB treatment significantly reduced PD-L1 expression in GL261-Luc2 cells when compared to mock treated cells (Figure 16).

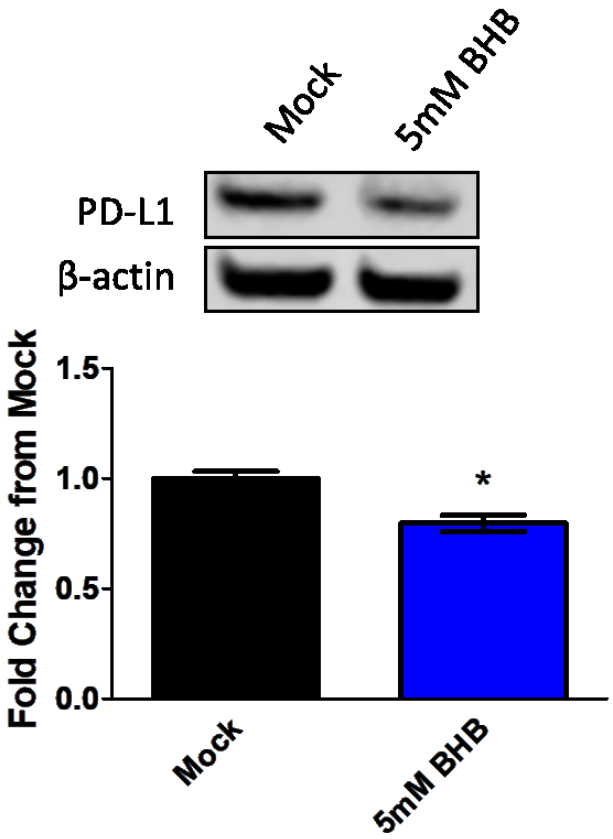


Figure 16: BHB reduces expression of PD-L1 on mouse malignant glioma cells. GL261-Luc2 cells were cultured in 5mM BHB for 24 hours prior to protein isolation. Western blotting analysis shows significantly decreased expression of PD-L1 (N=3; t-test; *p<0.05).

BHB does not impact activation of the PI3K pathway

Studies have shown that PD-L1 expression in tumor cell can be regulated by the PI3K-AKT-mTOR pathway [120, 216]. To determine the impact of BHB on this mechanism, the key components of this pathway were analyzed in GL261-Luc2 cells following treatment with different doses of BHB. Western blotting analysis shows that 5mM BHB did not significantly change the levels of phosphorylated PI3K, AKT or mTOR (Figure 17).

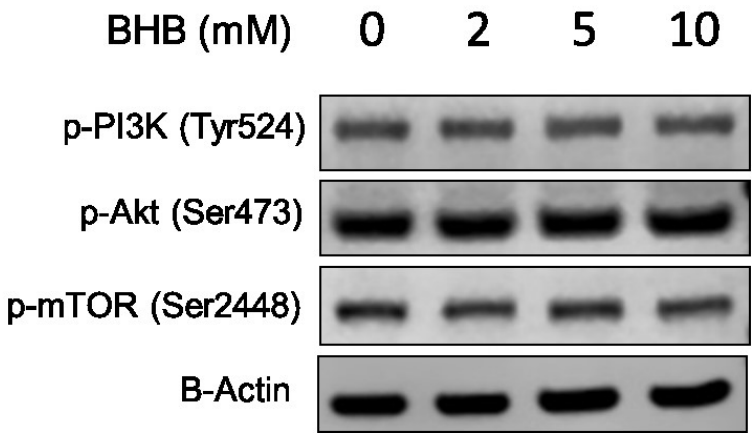


Figure 17: BHB does not significantly alter activation of the PI3K pathway. GL261-Luc2 cells were treated with 0, 2, 5 and 10mM BHB for 24 hours prior to protein isolation. Western blotting analysis was performed to assess levels of phospho-PI3K (Tyr524), p-Akt (Ser473), p-mTOR (Ser2448). B-actin was used as a loading control and the experiment was run in duplicate.

BHB does not impact IFN-γ-induced PD-L1 expression

Another mechanism of PD-L1 upregulation that has been described in glioma cells involves upregulation stimulated by interferon gamma (IFN-γ) [118]. The impact of BHB on this pathway was examined using GL261-Luc2 cells. Cells were treated with 5mM BHB prior to treatment with IFN-γ. Cells were stained with PD-L1 antibody and flow cytometry was performed. As expected, IFN-γ treatment alone significantly increased the expression of PD-L1 compared to mock treated cells. However, pretreatment with BHB prior to IFN-y treatment showed no significant change in PD-L1 expression (as measured by mean fluorescent intensity) when compared to IFN-γ treatment alone. The cells not treated with IFN-γ confirmed the result from Figure 16 above, demonstrating a significant decrease in PD-L1 expression with BHB treatment when compared to mock treated cells (Figure 18).

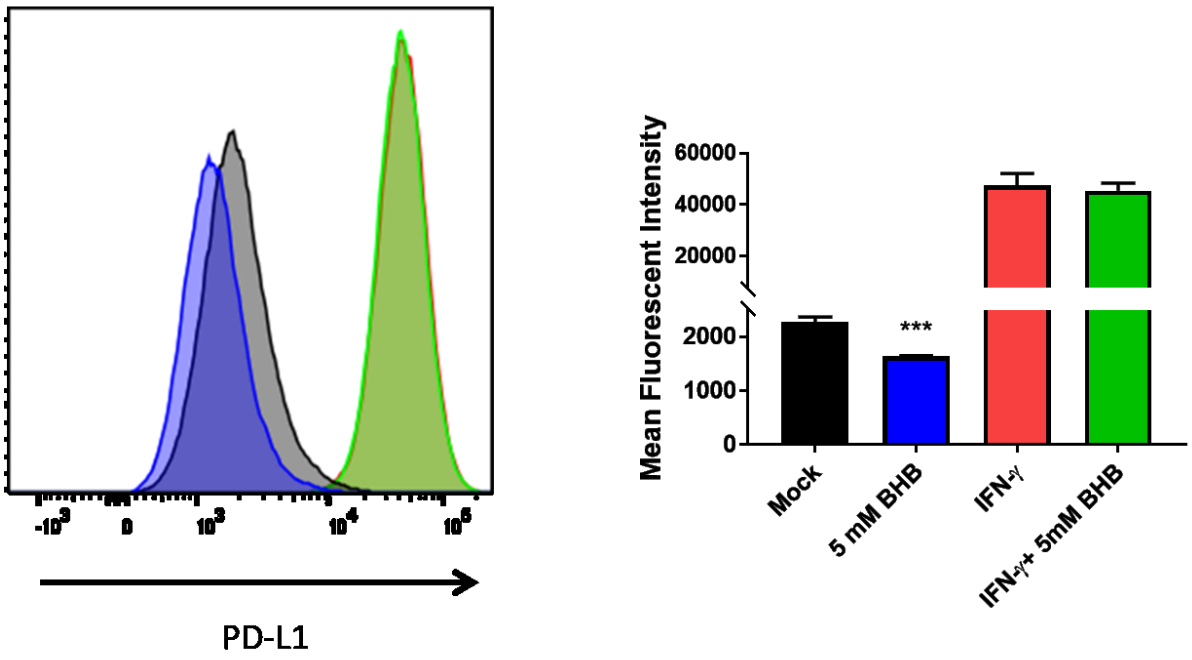


Figure 18: BHB does not impact IFN-γ-mediated PD-L1 upregulation. GL261-Luc2 cells were treated for 48 hours prior to treatment with IFN-γ (50 ng/mL) for 48 hours. Cells were stained with PD-L1 antibody and flow cytometry was performed. Experiments were run in triplicate and data is shown as representative histograms and mean fluorescent intensity of PD-L1 staining (N=3; t-test; ***p<0.001).

BHB does not impact PD-1, CTLA-4 or LAG3 expression on T cells

In *Chapter 2* we demonstrated that tumor-infiltrating CD8+ T cells from animals maintained on the KD had reduced expression of PD-1 and CTLA-4. To determine the impact of BHB on immune inhibitory checkpoint receptor expression, we used both CD8+ and CD4+ T cells isolated from P14 and SMARTA transgenic mice, respectively. T cells were treated with varying doses of BHB *in vitro* prior to flow cytometric analysis. There were no significant changes to PD-1, CTLA-4 or LAG-3 expression on either CD4+ or CD8+ T cells with any dose of BHB (Figure 19). Further, we found no significant changes in cell counts or viability in either T cell population following treatment with BHB (data not shown).

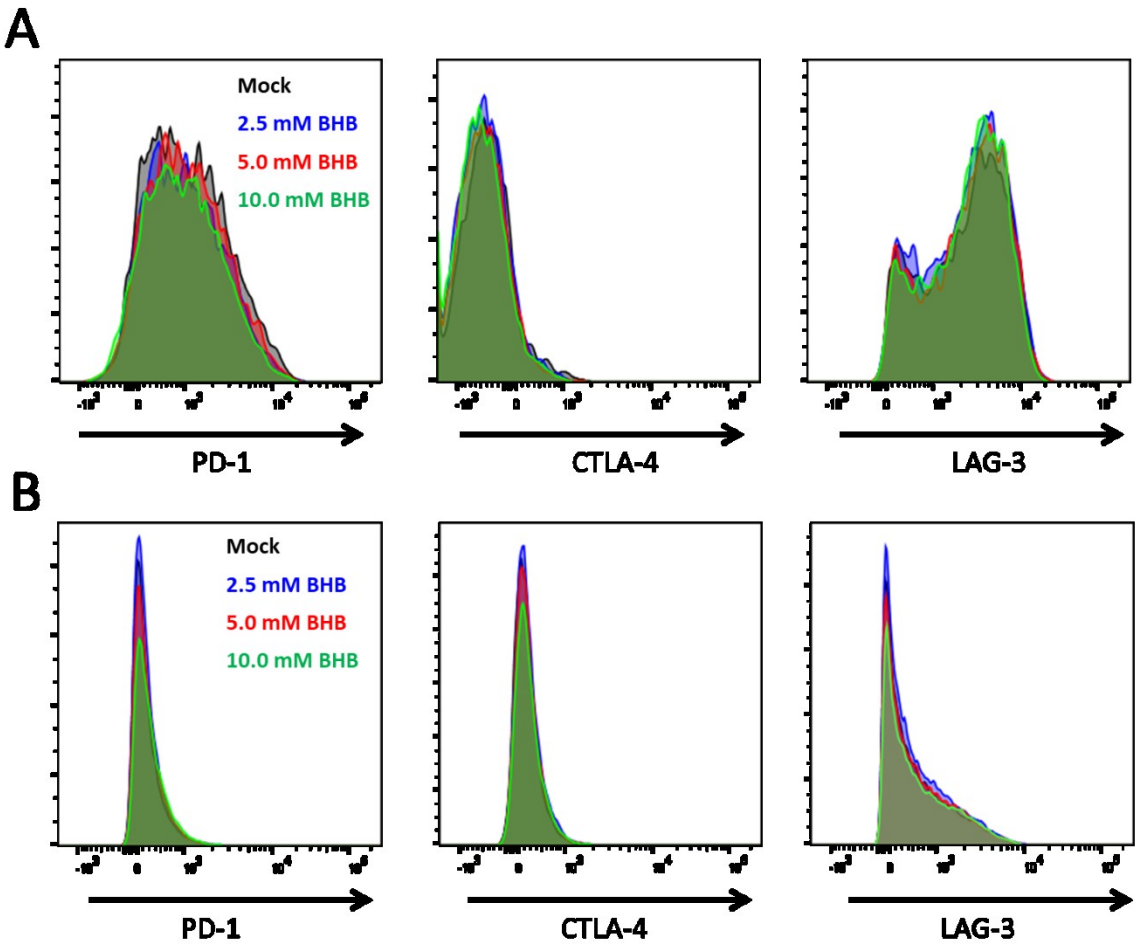


Figure 19: BHB does not impact immune checkpoint receptor expression in CD8 or CD4 T cells. CD8+ (A) and CD4+ T (B) cells were isolated from P14 and SMARTA transgenic mice, respectively. Cells were activated with their cognate LCMV peptide and IL-2 and treated with 0, 2.5, 5, and 10mM BHB every 48 hours for 8 days. Flow cytometry was performed to analyze PD-1, CTLA-4 and LAG-3 expression. Experiments were run in triplicate and data is shown as a representative histogram for each treatment group.

Impact of BHB on cytokine production in T cells

The ability to produce cytokines is a key indicator of effector function in T cells. In Chapter 2 we found that tumor-infiltrating CD8+ T cells from animals maintained on the KD produce significantly higher levels of IL-2, IFN-γ and TNF when stimulated with GL261-Luc2 cells. To assess the impact of BHB on cytokine production we again used CD8+ and CD4+ T cells isolated from P14 and SMARTA transgenic mice, respectively. T cells were treated with varying doses of BHB prior to analysis of cytokine levels in the media. In both CD8+ and CD4+ T cells there were no significant difference in levels of cytokines found in the media at any dose of BHB (Figure 20).

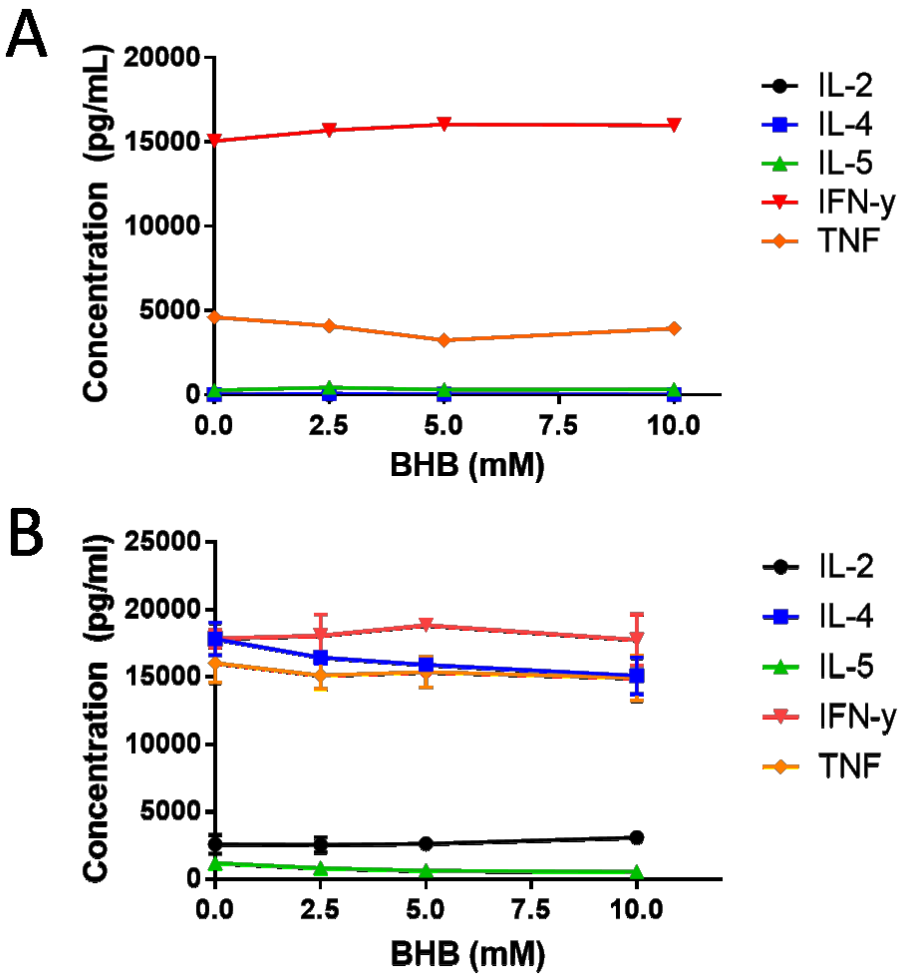


Figure 20: BHB does not alter secretion of cytokines by CD8+ and CD4+ T cells *in vitro*. CD8+ (A) and CD4+ T (B) cells were isolated from P14 and SMARTA transgenic mice, respectively. Cells were activated with their cognate LCMV peptide and IL-2 and treated with 0, 2.5, 5, and 10mM BHB every 48 hours for 8 days. On day 8, T cells were stimulated with IL-2, peptide and stimulator cells. A commercially available cytometric bead array (CBA) and flow cytometry was used to measure the presence of cytokines (IL-2, IL-4, IL-5, IFN-γ, and TNF) in the cell culture media.

DISCUSSION

Upregulation of PD-L1 on tumor cells is a key factor in immune escape of gliomas. We previously demonstrated in *Chapter 2* of this dissertation that glioma cells from mice maintained on KD exhibit decreased expression of PD-L1. There are two main mechanisms of upregulation: constitutively active oncogenic signaling pathways ("innate resistance") and interferon gamma (IFN-γ) mediated upregulation ("adaptive resistance"). It was demonstrated that phosphatase and tensin homolog (PTEN) deficient glioma cells exhibit greater PD-L1 expression and that this is driven in part by the PI3K-AKT-mTOR pathway [120, 216]. *In vivo s*tudies have shown that the KD is capable of impairing the mammalian target of rapamycin (mTOR) signaling pathway in a mouse model of epilepsy [217] and a glioma stem cell xenograft model [218]. The current study found that that treating glioma cells with BHB *in vitro* reduced expression of PD-L1 (Figures 16 and 18) suggesting that BHB may in part be acting on constitutive signaling pathways. However, there was no difference in the levels of activated effectors in the PI3K-AKT-mTOR pathway. Literature suggests that other constitutive oncogenic signal pathways such as JAK/STAT3 and EGFR/MAPK can mediate intrinsic induction of PD-L1 expression in other cancer cell lines [125]. While the impact of BHB on these pathways has not been explored, they represent important avenues for future studies.

IFN-γ is a proinflammatory cytokine produced mainly by activated T lymphocytes in response to antigen recognition during the adaptive immune response. Tumor-infiltrating lymphocytes (TILs), in response to recognition of tumor antigens, produce IFN-γ which can drive expression of PD-L1 in tumor cells [125]. This mechanism of tumor-mediated immune evasion termed "adaptive resistance" is supported by a study demonstrating that treatment with IFN-γ increases the surface expression of PD-L1 in

several glioma cell lines [118]. Recent studies show that IFN-γ-induced expression of PD-L1 may rely on activation of nuclear factor kappa B (NF-κB) [219]. We previously demonstrated that tumors from animals maintained on KD exhibited decreased activation of NF-κB [62] suggesting a possible mechanism by which KD could impair adaptive upregulation of PD-L1 expression. Fu *et al.* demonstrated that BHB treatment decreased activation of NF-κB and production of other pro-inflammatory mediators in immortalized mouse microglia cells [220]. However, the current study found that there was no difference in IFN-γ-meditated upregulation of PD-L1 when cells were pretreated with BHB, although the impact of BHB on NF-κB was not examined. This study may be limited by the fact that only one dose of INF-γ (50 ng/mL) was tested. This dose is a relatively high dose selected from the literature and that increased the intensity of PD-L1 expression in GL261-Luc2 cells 20-fold. It is possible that the effects of BHB on this pathway aren't extremely potent and may have been masked by the high dose of IFN-γ. Future studies not only need to explore the impact of BHB with different concentrations of IFN-γ but should also assess regulators of this pathway such as NF-κB.

Increased PD-1 expression is found on tumor-infiltrating T cells in multiple cancer types including glioma and indicates T cell exhaustion and reduced anti-tumor capabilities [221]. We previously demonstrated in *Chapter 2* that tumor-infiltrating CD8+ T cells from animals maintained on KD showed decreased expression of PD-1, increased anti-tumor cytokine (IL-2, TNF, IFN-γ) production and greater cytotoxic capabilities (data not shown) when cultured with tumor cells as compared to T cells isolated from control animals [127]. This suggests that KD may reduce exhaustion and improve function in glioma-infiltrating T cells; however; the role of BHB in this process is unknown.

The current study demonstrates that BHB does not change expression of immune checkpoint receptors including PD-1 in either CD8+ or CD4+ T cells *in vitro*. PD-1 expression can be regulated by a number of genetic and epigenetic mechanisms [221]. Studies show that high PD-1 expression on lymphocytes is sustained by high levels of FoxO1 transcription factor during chronic viral infection [222]. Conversely, the Tbet transcription factor antagonizes FoxO1 and inhibits expression of PD-1 [223]. It is possible that the KD shifts the balance away from FoxO1 expression and high PD-1 expression which is likely to be found in a T-cell-exhausted, immunosuppressed tumor microenvironment. It is difficult to recapitulate these conditions *in vitro* which may represent a limitation to the model chosen for the current study. To approach this issue we cultured T cells for a week prior to treating with BHB for an additional week to simulate treatment of "late" activated T cells. However at the end of the experiment, viability and the number of cells were too low to obtain quality data. Future studies will focus on further defining the impact of the KD on mechanisms of PD-1 regulation in the tumor microenvironment and may only be appropriate with *in vivo* studies.

In addition to the potential limitations of *in vitro* modeling mentioned above, it is also important to consider that these studies are using BHB treatment without the reduction of glucose. Although BHB has demonstrated anti-tumor benefits alone, the KD also elicits a decrease in blood glucose which may also contribute to the mechanisms discussed here. It is known that activated T cells rely heavily on glycolysis to support proliferation and efficient growth [151-154]. As discussed in *Chapter 2*, we do not yet know how this plays a role in the changes to T cells elicited by the KD, however; it may be appropriate to study the combination of reduced glucose with ketone treatment in future studies.

We previously demonstrated that the KD significantly alters PD-1 expression and cytokine production in tumor-infiltrating CD8+ T cells *in vivo* but did not elicit the same changes in a mouse model of acute and chronic LCMV infection [127]. This suggests that the impact of KD on lymphocytes may be specific to the tumor microenvironment and may help explain the results from the current study. It is has been shown that glioblastoma cells can secrete cytokines and other soluble factors that increase proliferation of T regulatory cells which can suppress anti-tumor immunity by inducing T cell anergy and apoptosis [224]. Evidence suggests that activation of NF-κB plays a key role in this ability of gliomas to manipulate the microenvironment [225]. Taken together with our data showing that KD reduces activation of NF-κB [62] this may provide an alternative explanation of KD-mediated influence on infiltrating lymphocytes.

It is also likely that the impact of the KD and BHB extends beyond T cells. For example, we demonstrated that KD enhanced IFN-γ and TNF production in tumor-infiltrating natural killer (NK) cells while reducing production of the immunosuppressive cytokine IL-10 in T regulatory cells [127]. Further, we found an increase in infiltrating CD4+ T cells but a decreased ratio of T regulatory cells (CD4+ FoxP3+) to CD4+ T cells in mice maintained on the KD compared to controls [127]. This decreased ratio reflected similar findings by Husain *et al.* in a study exploring KD in a pancreatic cancer model [226]. Though NK and T regulatory cells have important roles in the glioma microenvironment and may warrant further studies in the context of the KD and BHB, they are outside of the scope of this study.

METHODS

Tumor Cell Culture

GL261-Luc2 mouse malignant glioma cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum. Cells were cultured at 37^{o}C in 5% CO_2.

In Vitro T Cell Model

These experiments utilized both CD8+ and CD4+ T cells isolated from P14 and SMARTA transgenic mice, respectively. P14 mice are transgenic mice producing CD8+ T cells expressing T cell receptors specific for gp33-41 peptide from the lymphocytic choriomeningitis virus (LCMV). SMARTA mice are transgenic mice producing predominantly CD4+ T cells expressing T cell receptors specific for gp61-80 peptide from LCMV. This represents a simple well characterized *in vitro* model of T cell activation and function. These experiments were performed with the assistance and guidance of graduate student Kavita Manhas in Dr. Joseph Blattman's laboratory at ASU.

Treatments

Cells were treated with sodium (R)-3-hydroxybutyrate (Sigma-Aldrich, St. Louis, MO) diluted in water and brought to a final concentration in cell culture media. GL261-

Luc2 cells were treated with 50 ng/mL IFN-γ (Cell Signaling Technology, Danvers, MA) diluted in PBS.

Western Blotting

Protein was extracted from GL261-Luc2 cells using RIPA buffer (Cell Signaling Technology). Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Primary antibodies against PD-L1, phospho-PI3K (Tyr524), phospho-Akt (Ser473) and phospho-mTOR (Ser2448) (Cell Signaling Technology) were diluted 1:1000. Secondary antibodies were purchased from Licor Biosciences and were used at a dilution of 1:15,000. B-actin (Licor Biosciences, Lincoln, NE) was used as a loading control. Images were obtained using a Licor Odyssey Fc imager and data was analyzed using the Licor Image Studio software.

Immune Inhibitory Checkpoint Receptor Expression

CD8+ and CD4+ T cells were isolated from P14 and SMARTA transgenic mice, respectively. Cells were activated with their cognate LCMV peptide (1 μg/mL) and IL-2 (1.25×10^{-5} μg/mL) and treated with 0, 2.5, 5, and 10mM BHB every 48 hours for 8 days. Flow cytometry was performed following staining of cells with CD4, CD8, PD-1, CTLA-4 and LAG-3 antibodies (BD Biosciences, San Jose, CA). Viability and cell counts were recorded prior to flow cytometric analysis.

Cytometric Bead Array

T cells were cultured as described above for 8 days with 0, 2.5, 5 and 10mM BHB treatment. On day 8, T cells were restimulated with peptide and IL-2 for 12 hours prior to isolating conditioned media. A commercially available cytometric bead array (CBA) and flow cytometry was used to measure the presence of Th1/Th2 cytokines (IL-2, IL-4, IL-5, IFN-γ, and TNF) in the cell culture media.

Flow Cytometry

Analysis of cell populations and cytokine bead arrays were performed using an LSRFortessa flow cytometer (BD Biosciences, San Jose, CA). Flow cytometry data were analyzed with FlowJo10 (Tree Star Inc., Ashland, OR) and graphs were generated using Prism 7 software (GraphPad Software, La Jolla, CA).

CHAPTER 5

THE KETOGENIC DIET INCREASES THE EXPRESSION OF MICRORNAS PREDICTED TO TARGET PD-L1

INTRODUCTION

The upregulation of PD-L1 plays a large role in immune evasion by gliomas. As discussed in *Chapter 4* there are multiple signaling pathways that can regulate PD-L1 expression. This upregulation can be mediated by adaptive induction by interferon gamma (IFN-γ) and/or intrinsic induction by oncogenic signaling pathways; however additional mechanisms including responses to the hypoxic microenvironment and microRNA regulation have also been implicated [125]. Taken together the literature suggests complicated regulation of PD-L1 expression which warrants a further look at the impact of the ketogenic diet (KD) on these additional mechanisms.

MicroRNAs (miRNAs) are short, non-coding RNA molecules that regulate gene expression by suppressing mRNA translation and reducing mRNA stability [9]. miRNA dysregulation has emerged as a crucial driver of cancer biology [227]. Alterations in miRNA processing machinery [228] and global reduction in expression of miRNAs [229] have been described in many cancers. The interaction between tumors and the cells of the immune system are also influenced by miRNAs [230]. There is now substantial evidence in a variety of cancer cell types including glioma, showing that microRNAs can impact expression of PD-L1 by either targeting it directly or targeting upstream effectors in its regulation [231]. This may be a potential mechanism by which the KD impacts expression of PD-L1.

Support for the hypothesis that the KD may impact expression of miRNAs comes from the study demonstrating that β-hydroxybutyrate (BHB), the major ketone elevated in the blood as a result of the ketogenic diet can also inhibit histone deacetylases (HDACs). HDACs are critical regulators of gene expression that enzymatically remove the acetyl group from histones, thus facilitating transcriptional repression. HDAC inhibitors are actively being tested for their ability to reverse the abnormal gene expression patterns inherent to the cancer epigenome which includes releasing transcriptional repression of anti-tumor microRNAs in a variety of cancers [13, 14]. A number of *in vitro* and *in vivo* studies have demonstrated the ability of chromatin-modifying drugs such as HDAC inhibitors to alter miRNA expression in cancer and has been reviewed thoroughly by Ali *et al.* [232]. The HDAC-inhibiting properties of BHB was first demonstrated in normal untransformed cells by Shimazu et al [53] but has only recently been demonstrated in the context of glioma cells which was demonstrated in *Chapter 3*. Given the evidence that the KD may work in part by epigenetic mechanisms, it was postulated that the KD may alter the expression of miRNAs that target expression of PD-L1.

In this study we collaborated with Dr. Nelofer Syed at the Imperial College of London who performed RNA-sequencing on tumor tissue from animals maintained on the KD to assess the changes in miRNA expression. This data shows that the KD does in fact alter the expression of several microRNAs in glioma bearing-mice. miRNA target prediction software was then used to identify miRNAs that target the immune inhibitory checkpoint ligand PD-L1. Using these predictions as well as published studies from the literature we've identified multiple candidate miRNAs that may help explain the changes to PD-L1 expression described in *Chapter 2* of this document and inform future studies. While the scope of this study is limited to PD-L1, this data offers a potential mechanism

that may be useful in describing the KDs impact on additional hallmarks of cancer with goal of better understanding the pluripotent nature of this approach.

RESULTS

The KD increases blood BHB, reduces blood glucose and slows tumor growth in vivo

Tumor-bearing mice maintained on the ketogenic diet (KD) had significantly elevated blood BHB levels and reduced circulating blood glucose (Figure 21 A and B). Animals maintained on the KD also showed slower tumor growth rates as measured by bioluminescent imaging (Figure 21C). These results are consistent with work previously published in our laboratory [61, 62, 80, 127] as well as unpublished data.

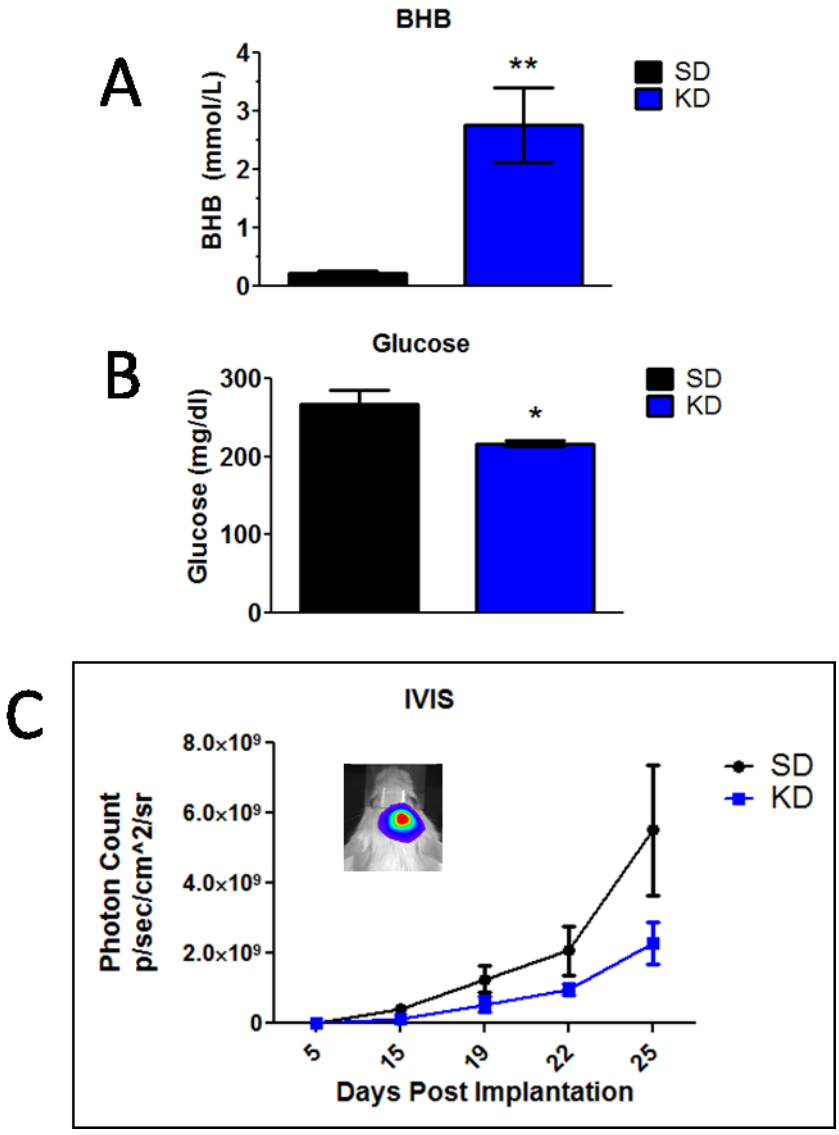


Figure 21: The KD increases blood BHB, decreases blood glucose, and slows tumor growth *in vivo*. Blood ketone and glucose measurements show (A) higher BHB and (B) lower glucose in KD treated animals (C) bioluminescent tumor signals plotted as *in vivo* photon count versus days post-implantation. (N = 5; *p < 0.05; **p < 0.01; ***p < 0.001).

KD increases significantly alters expression of several microRNAs

RNAseq analysis was performed by our collaborator Dr. Nelofer Syed from the Imperial College of London on tumor tissue from animals treated with the KD versus those maintained on a standard rodent diet. These results demonstrate that the KD significantly increases expression of several miRNAs (Figure 22), many of which have demonstrated tumor suppressor properties in glioma [233]. Only statistically significant changes in expression were included in this graph.

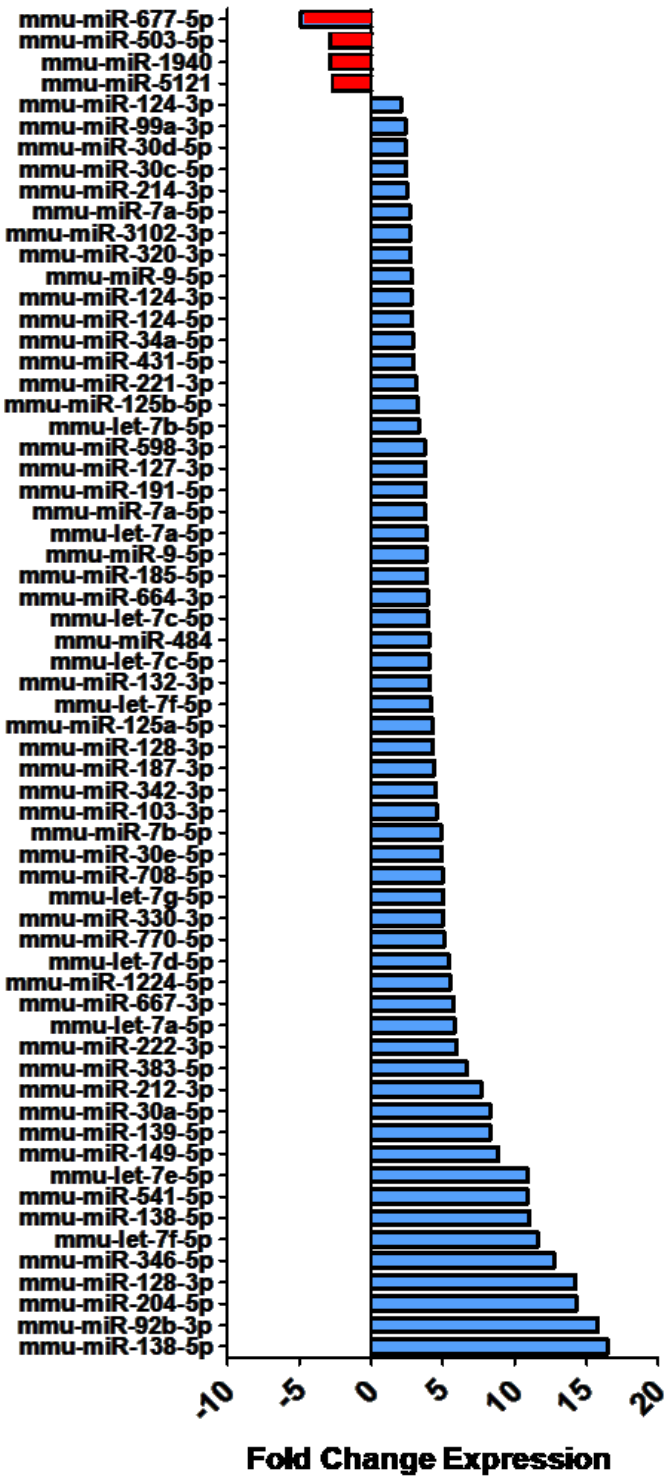
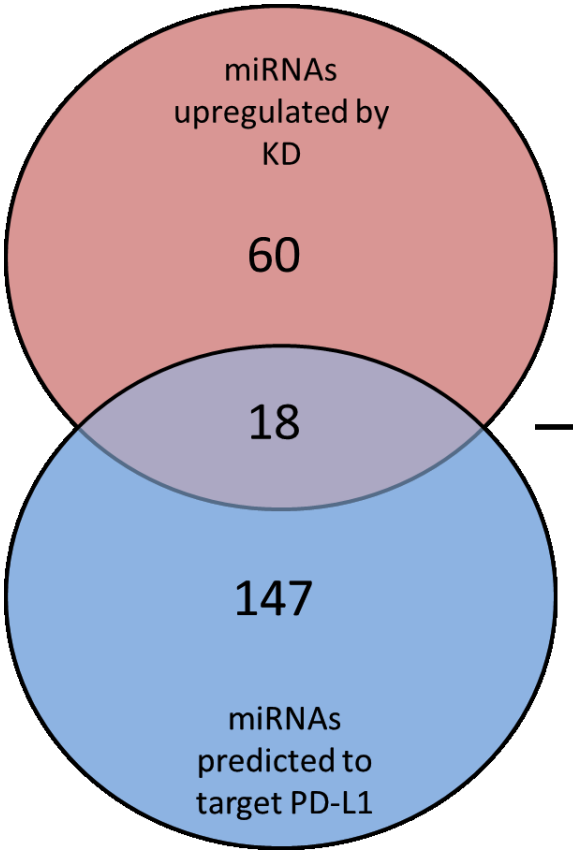


Figure 22: The KD significantly alters the expression of multiple miRNAs. RNA-sequencing analysis was performed on tumors from animals maintained on the KD and compared with those maintained on a standard rodent diet. Data is represented as a fold change in expression from standard diet fed controls (N=5).

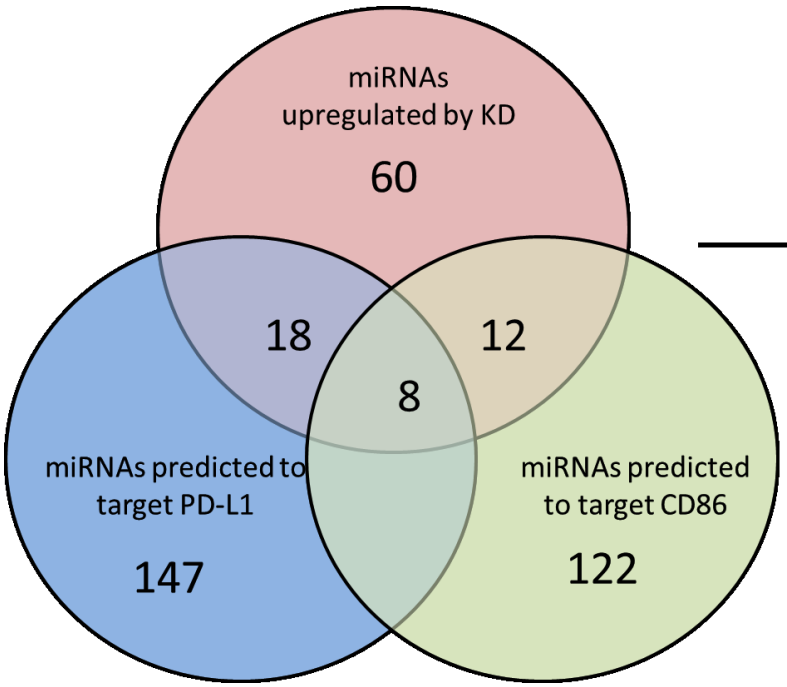
The KD increases expression of miRNAs predicted to target PD-L1

It was demonstrated in *Chapter 2* that tumors from animals maintained on the KD showed reduced expression of the immune inhibitory ligand PD-L1. In an attempt to connect the results of that study to the miRNA changes found in the current study, miRanda target prediction software was used to generate a list of miRNAs predicted to target PD-L1. Out of the 147 miRNAs predicted to target PD-L1, 18 were also found to be upregulated in the tumors from animals maintained on the KD (Figure 23). Single miRNAs often bind and regulate multiple target genes. The study in *Chapter 2* also demonstrated that the KD reduced CD86 expression which is another immune inhibitory checkpoint ligand. To determine if any of the 18 candidates also target CD86, miRNAs predicted to bind CD86 were compared those predicted to target PD-L1. The list was narrowed to 8 miRNAs significantly upregulated by the KD that are predicted to target both PD-L1 and CD86 (Figure 24).



miRNA matches	fold change upregulation in KD
mmu-let-7a	3.86
mmu-let-7a	5.82
mmu-let-7b	3.32
mmu-let-7c	4.11
mmu-let-7c-2	4.03
mmu-let-7d	5.39
mmu-let-7f	4.14
mmu-let-7f	11.59
mmu-let-7g	5.02
mmu-miR-103	4.56
mmu-miR-1224	5.50
mmu-miR-124	2.84
mmu-miR-204	14.28
mmu-miR-214	2.50
mmu-miR-34a	2.92
mmu-miR-383	6.65
mmu-miR-484	4.04
mmu-miR-667	5.75

Figure 23: The KD increases the expression of multiple miRNAs predicted to target PD-L1. miRanda target prediction software was used to identify miRNAs predicted to target PD-L1. These predictions were then compared to the list of miRNAs significantly upregulated in tumors from animals maintained on the KD.



miRNAs targeting PD-L1 and CD86	fold change upregulation in KD
mmu-let-7a	5.8
mmu-let-7b	3.3
mmu-let-7c	4.1
mmu-let-7d	5.4
mmu-let-7e	10.9
mmu-let-7f	11.6
mmu-let-7g	5.0
mmu-miR-484	4.0

Figure 24: The KD increases expression of miRNAs predicted to target both PD-L1 and CD86. miRanda target prediction software was used to identify miRNAs predicted to target both immune inhibitory checkpoint ligands PD-L1 and CD86. These predictions were then compared to the list of miRNAs significantly upregulated in tumors from animals maintained on the KD.

DISCUSSION

Advances in our understanding of the molecular biology of GBM have revealed that non-coding RNAs such as miRNAs are often dysregulated in GBM and many other cancers. This knowledge has led to the increased interest in leveraging miRNAs for cancer therapy and biomarker development [234]. In the present study, we found that the KD significantly alters the expression of several miRNAs in a mouse model of malignant glioma, many of which have been implicated in multiple hallmarks of cancer. miRNA target prediction software was used to determine which of these miRNAs may target PD-L1 expression and 18 candidates were identified. Some of these candidates have published interactions with PD-L1 and the immune system. For example miR-34a has been shown to directly target PD-L1 expression in glioma cells [235] and non-small cell lung cancer (NSCLC) cells [236, 237]. Others on the list such as the let-7 family of miRNAs have demonstrated tumor suppressor functions in several cancers including GBM [238] but no published interaction with PD-L1. Finally there are candidates on the list that have yet to be explored in the context of cancer and may be represent novel avenues for future studies.

The most highly upregulated miRNA in tumors from animals maintained on the KD was miR-138. In multiple cancers including GBM, miR-138 has been shown to be downregulated and acts as a tumor suppressor [239-244]. Qiu et al showed that increased expression of miR-138 is associated with longer overall and progression-free survival in GBM patients [241]. While miR-138 targets many different genes involved in various hallmarks of cancer, there is also evidence showing it directly impacts immune inhibitory checkpoint pathways. For example a recent study demonstrated that miR-138 directly targets PD-L1 in colorectal cancer cells [245]. The *in vivo* data from *Chapter 2*

showed significantly reduced expression of PD-L1 the tumors from animals maintained on the KD. Further, our collaborator Dr. Nelofer Syed has demonstrated that BHB treatment *in vitro* leads to significant upregulation of miR-138 in a human glioma cell line (unpublished data). Taken together this data suggests that the KD, due in part to BHB, may alter PD-L1 expression in glioma cells by upregulating miR-138.

Mounting evidence demonstrates that tumors can influence with their microenvironment by secreting miRNAs [246]. Tumor immunity can be modulated by miRNAs and other non-coding RNAs that are transferred between tumor cells and immune cells in tumor microenvironments via exosomes or other microvesicles [230]. This suggests that the KD's impact on miRNA expression in tumors may impact also impact T cells and other cells in the glioma microenvironment. Wei *et al* showed that intravenous administration of miR-138 in GL261-implanted mice slowed tumor growth and demonstrated significant downregulation of CTLA-4, PD-1, and FoxP3 on T cells in the tumor microenvironment [247]. Another study using the GL261 mouse glioma model demonstrated that miR-124, which is also commonly downregulated in GBM, slowed tumor growth and led to the increased production of IL-2, IFN-γ and TNF in tumor-infiltrating lymphocytes indicating increased activation and effector function [248]. Interestingly both miR-138 and miR-124 were significantly upregulated in the tumors from animals maintained on the KD (Figure 14) our results from the study described in *Chapter 2* describing the impact of the KD on T cells parallel the results of these two studies. It could therefore be postulated that the KD may lead to the increase in expression of miRNAs such as miR-138 or miR-124 in tumor cells which are then secreted into the microenvironment and impact immune function. Further studies exploring the ability of the KD to impact the microenvironment via secreted miRNAs are warranted.

miRNAs often have several target mRNAs and as literature and prediction software shows, multiple miRNAs can target the same mRNA impacting multiple aspects of tumor biology [249]. This lends to the complex nature of miRNA regulation of gene expression but it may also help us better understand the pluripotent nature of the KD. While the focus of this chapter is specifically on PD-L1 and immune modulation, the miRNAs altered by the KD target several different hallmarks of cancer (Table 2) [249-254]. In addition, some of these miRNAs such as miR-138 and miR-34a (described above), target not only PD-L1 but other genes that have we've explored in the context of the KD. For example, miR-138 was shown to directly target a key effector in the response to hypoxia, hypoxia-inducible factor 1 α (HIF-1α) [240]. We previously demonstrated in a mouse model of malignant glioma that tumors from animals maintained on the KD have significantly reduced expression of HIF-1α [62]. Another example is miR-34a which targets PD-L1 as well as the key effector in DNA damage repair, RAD51 [255]. RAD51 is crucial for homologous recombination repair of double-stranded DNA breaks and is overexpressed in several human cancers [180, 181]. The increased expression of RAD51 in cancer leads to resistance to double-stranded break inducing anti-cancer therapies [182-184]. We have demonstrated a reduction in RAD51 protein expression in a variety of malignant glioma cells following treatment with BHB *in vitro* and in tumor tissue from animals maintained on a KD (*Chapter 3*). While miR-138 and miR-34a offer two specific examples of crossover pathways, several additional miRNAs on this list can potentially be connected to previous studies.

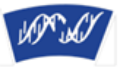
Hallmark Symbol	Targeted Hallmark of Cancer	miRNA(s) Up-Regulated with KD
	Sustaining Proliferative Signaling	miR-185-5p, let-7e-5p, miR-770-5p, miR-103-3p, miR-124-3p, miR-34a-5p, miR-320-3p, miR-125a-5p, miR-125b-5p, miR-342-3p, miR-667-3p, miR-9-5p, miR-128-3p
	Evading Growth Suppressors	miR-7b-5p, miR-7a-5p, miR-431-5p, miR-9-5p, miR-128-3p, miR-664-3p, miR-30a-5p, miR-30e-5p, miR-30c-5p, miR-30d-5p
	Avoiding Immune Destruction	miR-30a-5p, miR-30e-5p, miR-30c-5p, miR-30d-5p, miR-124-3p, miR-124-5p, miR-34a-5p, miR-128-3p, miR-7a-5p, miR-125b-5p
	Enabling Replicative Immortality	miR-342-3p, let-7 family, miR-34a-5p
	Tumor-Promoting Inflammation	let-7f-5p, let-7a-5p, let-7g-5p, let-7c-5p, let-7b-5p, miR-128-3p
	Activating Invasion & Metastasis	miR-664-3p, miR-124-3p, miR-128-3p, miR-30a-5p, miR-30e-5p, miR-30c-5p, miR-30d-5p, miR-666-3p, miR-346-5p
	Inducing Angiogenesis	miR-124-3p, miR-431-5p, miR-128-3p, miR-664-3p, miR-383-5p
	Genome Instability & Mutation	miR-103-3p, miR-204-5p
	Resisting Cell Death	let-7f-5, let-7e-5p, let-7a-5p, let-7g-5p, let-7c-5p, let-7b-5p, miR-342-3p, miR-92b-3p, miR-139-5p, miR-103-3p, miR-9-5p, miR-124-3p
	Deregulating Cellular Energetics	miR-125a-5p, miR-342-3p, miR-9-5p, miR-204-5p, mir-185-5p, miR-128-3p, miR-99a-3p

Table 2: miRNAs upregulated by the KD target multiple hallmarks of cancer. miRNAs upregulated in tumors from animals maintained on the KD were compared to literature validating the role of specific miRNAs targeting various hallmarks of tumor growth and progression [249-254]. Symbols adapted from Hanahan and Weinberg *et al.* [7].

The current study is far from validation of the roles specific miRNAs play in the anti-tumor effects of the KD. It does however provide a framework for future studies and may help us better explain our previous results. In addition to the several specific changes elicited by the KD discussed throughout this document, studies done in our laboratory using a mouse model of malignant glioma demonstrated that the KD exerts a global effect on the aberrant genetic landscape found in tumors [50]. Gene expression was compared in tumor tissue and tissue from the contralateral non-tumor containing side of the brain using cDNA array technology. This work showed that overall gene expression in tumor from animals fed the KD was shifted more towards the gene expression found in non-tumor containing tissue from animals fed either the KD or standard diet [61]. It is possible that the KD may alter global gene expression by altering the expression of several miRNAs. To our knowledge the current study provides the first evidence suggesting this, which moves us closer to understanding more completely the mechanisms underlying the KDs anti-tumor effects.

METHODS

Mice and tumor implantation

GL261-Luc2 cells were harvested and implanted into mice as previously described in the methods section of *Chapter 2* in this dissertation document and previous publications [62, 80, 127, 158].

Treatment and Animal Monitoring

Following implantation surgery, animals were fed standard rodent chow for 3 days. Animals were then randomized to remain on standard diet (SD) or changed to a KD (KetoCal®; Nutricia North America, Gaithersburg, MD). The KD was obtained directly from the manufacturer and is a nutritionally complete diet providing a 4:1 ratio of fats to carbohydrates plus protein (72% fat, 15% protein, and 3% carbohydrate). The KD was prepared by mixing KetoCal® with water (2:1) and fed to the animals each day (ad libitum). Bioluminescence was analyzed to quantify tumor burden as described [80] Serum β-hydroxybutyrate (BHB) and glucose levels were measured using a Precision Xtra® blood monitoring system (Abbott Laboratories, Abbott Park, IL).

Tissue Harvesting

On Day 25 post-implantation, animals were euthanized and tumor tissue was snap frozen in liquid nitrogen.

RNA isolation and miRNA Sequencing

Flash frozen tumor samples were shipped to the laboratory of Dr. Nelofer Syed at the Imperial College in London. RNA was isolated and RNA-sequencing was performed.

miRNA Prediction Analysis

miRanda target prediction software was used to identify mouse and human miRNAs targeting mouse PD-L1 and CD86 [256-259]. These predictions were validated using TargetScan [260] and RNA22 [261] miRNA-target prediction programs.

CHAPTER 6

DISCUSSION

Glioblastoma multiforme (GBM) is the most aggressive primary cancer of the brain. Despite surgery followed by radiation and chemotherapy, these patients have a median survival time of ~15 months with a less than 6% five-year survival rate [262]. Complete surgical resection is precluded by the highly infiltrative nature of malignant brain tumors and the critical functions of the brain. Further, currently available therapies can kill most, but not all of the cells that remain after surgery, thus providing a seed for the formation of a recurrent tumor, typically within 2 years of their original diagnosis and in the same general area as the primary tumor. These tumors are often resistant to additional therapy with the same chemotherapy agents, and the use of additional radiation increases the risk of damage to adjacent normal tissue. In addition, once a tumor recurs following chemotherapy with temozolomide, there are few additional chemotherapeutic agents with demonstrated efficacy for the treatment of brain tumors. Advances in survival and quality of life rely on new therapeutic strategies, especially those that can enhance the efficacy of current treatment options. Despite decades of preclinical research and clinical trials the dismal prognosis for these patients remains largely unchanged. Novel, innovative therapeutic approaches are needed to improve patient outcomes.

Evasion of the anti-tumor immune response has emerged as a crucial enabling characteristic of GBM. In particular, the ability of GBM to take advantage of immune inhibitor checkpoint pathways such as PD-1/PD-L1 is actively being explored as a therapeutic approach. For example, there have been a number of clinical trials initiated to determine the effectiveness of PD-1 checkpoint blockade in GBM [125]. While this

approach has yet to be tested thoroughly in clinical trials, the PD-1 inhibitor nivolumab was recently proven ineffective as a monotherapy in recurrent GBM patients. Treatment with nivolumab alone failed to improve overall survival in recurrent GBM patients when compared to standard of care which is bevacizumab in a phase 3 trial (NCT02017717; KEYNOTE 028; ClinicalTrials.gov). This may reflect the mounting preclinical evidence suggesting that combining PD-1 blockade with additional checkpoint blockade or other immunotherapeutic strategies is more effective than monotherapy [141, 142, 263].

In *Chapter 2* we investigated the impact of the KD on the glioma-reactive immune response *in vivo*. We demonstrate that mice fed the KD had increased tumor-reactive innate and adaptive immune responses, including increased cytokine production and cytolysis via tumor-reactive CD8+ T cells. Additionally, we saw that mice maintained on the KD had increased CD4+ infiltration, while T regulatory cell numbers stayed consistent. Lastly, mice fed the KD showed a significant reduction in expression of the immune inhibitory receptor PD-1 on T cells as well as decreased expression of the corresponding inhibitory ligand PD-L1 on glioma cells. These results suggest that the KD may work in part as an immune adjuvant, boosting tumor-reactive immune responses in the microenvironment by alleviating immune suppression. This evidence suggests that the KD increases tumor-reactive immune responses, and may have implications in combinational treatment approaches.

To our knowledge this is the first published study to explore the KD in the context of anti-tumor immunity and demonstrate non-pharmalogical disruption of immune inhibitory checkpoint pathways; however, whether or not this results in better response to immune checkpoint blockade therapy has yet to be determined and warrants future studies. Although showing some preclinical efficacy, single agent checkpoint blockade therapy may be limited by redundant and compensatory mechanisms. For example,

Huang et al demonstrated compensatory upregulation of PD-1 and CTLA-4 expression when using single-agent checkpoint blockade in metastatic ovarian cancer [264]. Preclinical evidence shows that combinatorial checkpoint blockade for glioma is more effective that any single immune checkpoint inhibitor alone [141, 142]. While the main focus of this proposal is the PD-1 checkpoint we have also demonstrated that the KD reduces expression of the cytotoxic T lymphocyte antigen-4 (CTLA-4) receptor on T cells and its corresponding ligand CD86 on glioma cells (*Chapter 2*) which is an additional immune inhibitory checkpoint mechanism and the focus of therapeutic development [133]. This suggests that the KD may potentially enhance the efficacy of immune checkpoint blockade strategies by reducing the expression of multiple inhibitory receptors/ligands and impairing compensatory upregulation. Further studies examining the impact of KD on these mechanisms are needed.

While this dissertation document focuses mainly on the PD-1/PD-L1 pathway, there are additional promising immunotherapeutic strategies that may worth exploring in combination with KD. As mentioned above, therapeutics targeting CTLA-4 have shown promise alone and in combination with PD-1 and PD-L1 inhibitors and may be more effective when given in combination with KD. Another target in development is indoleamine 2,3 dioxygenase 1 (IDO1), which has emerged as another important immunosuppressive mechanism in gliomas and has led to the development of IDO1 inhibitors, may be regulated by similar pathways driving PD-L1 expression [265]. IDO1 inhibitors have shown promise in preclinical studies especially in combination with immune inhibitory checkpoint inhibitors [141] and temozolomide [266]. Further studies exploring KD in combination with IDO1 inhibition and additional novel immunotherapeutic strategies including may be warranted.

While the KD may impact tumor-mediated immune suppression, this is not the only anti-tumor mechanism to consider. Our laboratory previously published a study demonstrating that the KD greatly enhances survival in a mouse model of malignant glioma when combined with radiation [80]. The *in vitro* studies described in *Chapter 3* suggest that BHB may play a large role in KD-mediated radiosensitization by inhibiting HDACs and impairing radiation-induced DNA damage repair capabilities in glioma cells. As radiation-induced tumor cell killing is known to be immunogenic, it is possible that the KD also indirectly enhances the anti-tumor immune response by augmenting the effect of radiation. The interactions between radiation and the immune system have been investigated, with several studies describing the synergistic effects on local and distant tumor control when radiation therapy is combined with immunotherapy [167, 168]. Interestingly, several recent studies now have suggested that PD-L1 expression may be linked to radiation response in cancer [267-272], providing another interesting avenue linking the radiation and immune enhancing properties of the KD. Taken together, the data described in each of the studies in this dissertation suggest that the KD may exert multiple anti-tumor pressures simultaneously, thus making it an attractive adjuvant to both standard of care and immunotherapies.

In *Chapter 4* we investigated the impact of BHB on PD-L1 expression on glioma cells and PD-1 expression on T cells. We show that BHB treatment of glioma cells *in vitro* leads to a reduction in PD-L1 expression. However, BHB failed to change expression of the immune inhibitory receptors including PD-1 on T cells *in vitro* suggesting an alternative mechanism may explain the *in vivo* results from *Chapter 2*. These results may also warrant further *in vitro* studies to combine reduced glucose conditions with BHB treatment to more accurately recapitulate the conditions of a full KD. To our knowledge this is the first study examining the impact of BHB on the PD-1/PD-L1

pathway and represents a novel mechanism underlying the KD and its ability to alter the way tumor cells interact with the immune system. This data lends to the growing body of evidence from our laboratory and others demonstrating that BHB acts as more than a simple replacement energy substrate and may drive many of the anti-tumor effects of the KD [79]. This work supports the growing idea that ketones may be effective in supplementing the rigors of a full ketogenic diet or perhaps replacing it.

In *Chapter 5*, we investigated the impact of the KD on expression of miRNAs in collaboration with the Syed Laboratory and found that the tumors from animals maintained on the KD have significantly increased expression of several different miRNAs. Screening this list we find that several of these miRNAs are predicted to target PD-L1, some of which have already been experimentally validated in previously published studies. This data suggest one potential mechanism by which the KD may act on immune inhibitory checkpoint pathways. This was supported by work done in the laboratory of our collaborator Dr. Nelofer Syed showing that treatment of glioma cells with BHB *in vitro* upregulates expression of miR-138 (unpublished data), which is a tumor-suppressor miRNA and targets PD-L1 in cancer cells [245] as well as PD-1 and other immune checkpoint pathways in T cells [247]. Future studies connecting the upregulation of miR-138 and other miRNAs by KD-mediated changes in PD-1 or PD-L1 expression are warranted. However, as discussed in *Chapter 4* there are multiple pathways beyond tumor-immune interaction that are impacted by the miRNAs upregulated by the KD which may reveal a broader mechanism by which the KD impacts multiple hallmarks of cancer.

As interest in using the KD for brain tumor patients continues to grow rapidly, greater understanding of the mechanisms underlying this approach are needed, particularly as they pertain to its potential to augment other therapies. The work in this

dissertation is the first to describe the impact of the KD on the anti-glioma immune response and thus provides a foundation for better understanding how it may work in combination with immunotherapeutic strategies. Taken together with other studies conducted in our laboratory, this work contributes significantly to the overall goal of developing the ketogenic diet as an effective adjuvant strategy for malignant glioma.

REFERENCES

1. Cai X, Sughrue ME: **Glioblastoma: new therapeutic strategies to address cellular and genomic complexity**. *Oncotarget* 2018, **9**(10):9540-9554.

2. Warburg O, Wind F, Negelein E: **THE METABOLISM OF TUMORS IN THE BODY**. *The Journal of general physiology* 1927, **8**(6):519-530.

3. Wolf A, Agnihotri S, Guha A: **Targeting metabolic remodeling in glioblastoma multiforme**. *Oncotarget* 2010, **1**(7):552-562.

4. Nijsten MW, van Dam GM: **Hypothesis: using the Warburg effect against cancer by reducing glucose and providing lactate**. *Medical hypotheses* 2009, **73**(1):48-51.

5. Cantor JR, Sabatini DM: **Cancer cell metabolism: one hallmark, many faces**. *Cancer discovery* 2012, **2**(10):881-898.

6. Ward PS, Thompson CB: **Metabolic reprogramming: a cancer hallmark even warburg did not anticipate**. *Cancer cell* 2012, **21**(3):297-308.

7. Hanahan D, Weinberg RA: **Hallmarks of cancer: the next generation**. *Cell* 2011, **144**(5):646-674.

8. Lewis NE, Abdel-Haleem AM: **The evolution of genome-scale models of cancer metabolism**. *Frontiers in physiology* 2013, **4**:237.

9. Obre E, Rossignol R: **Emerging concepts in bioenergetics and cancer research: metabolic flexibility, coupling, symbiosis, switch, oxidative tumors, metabolic remodeling, signaling and bioenergetic therapy**. *The international journal of biochemistry & cell biology* 2015, **59**:167-181.

10. Miller DM, Thomas SD, Islam A, Muench D, Sedoris K: **c-Myc and cancer metabolism**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2012, **18**(20):5546-5553.

11. Dibble CC, Cantley LC: **Regulation of mTORC1 by PI3K signaling**. *Trends in cell biology* 2015, **25**(9):545-555.

12. Courtnay R, Ngo DC, Malik N, Ververis K, Tortorella SM, Karagiannis TC: **Cancer metabolism and the Warburg effect: the role of HIF-1 and PI3K**. *Molecular biology reports* 2015, **42**(4):841-851.

13. Roberts DJ, Miyamoto S: **Hexokinase II integrates energy metabolism and cellular protection: Akting on mitochondria and TORCing to autophagy**. *Cell death and differentiation* 2015, **22**(2):248-257.

14. Martini M, De Santis MC, Braccini L, Gulluni F, Hirsch E: **PI3K/AKT signaling pathway and cancer: an updated review**. *Annals of medicine* 2014, **46**(6):372-383.

15. Marie SK, Shinjo SM: **Metabolism and brain cancer**. *Clinics (Sao Paulo, Brazil)* 2011, **66 Suppl 1**:33-43.

16. Tarrado-Castellarnau M, de Atauri P, Cascante M: **Oncogenic regulation of tumor metabolic reprogramming**. *Oncotarget* 2016, **7**(38):62726-62753.

17. Justus CR, Sanderlin EJ, Yang LV: **Molecular Connections between Cancer Cell Metabolism and the Tumor Microenvironment**. *International journal of molecular sciences* 2015, **16**(5):11055-11086.

18. Pore N, Jiang Z, Shu HK, Bernhard E, Kao GD, Maity A: **Akt1 activation can augment hypoxia-inducible factor-1alpha expression by increasing protein translation through a mammalian target of rapamycin-independent pathway**. *Molecular cancer research : MCR* 2006, **4**(7):471-479.

19. Wei W, Shi Q, Remacle F, Qin L, Shackelford DB, Shin YS, Mischel PS, Levine RD, Heath JR: **Hypoxia induces a phase transition within a kinase signaling network in cancer cells**. *Proceedings of the National Academy of Sciences of the United States of America* 2013, **110**(15):E1352-1360.

20. Corbet C, Feron O: **Metabolic and mind shifts: from glucose to glutamine and acetate addictions in cancer**. *Current opinion in clinical nutrition and metabolic care* 2015, **18**(4):346-353.

21. Casey SC, Amedei A, Aquilano K, Azmi AS, Benencia F, Bhakta D, Bilsland AE, Boosani CS, Chen S, Ciriolo MR *et al*: **Cancer prevention and therapy through the modulation of the tumor microenvironment**. *Seminars in cancer biology* 2015, **35 Suppl**:S199-s223.

22. Fruehauf JP, Meyskens FL, Jr.: **Reactive oxygen species: a breath of life or death?** *Clinical cancer research : an official journal of the American Association for Cancer Research* 2007, **13**(3):789-794.

23. Weinberg F, Chandel NS: **Reactive oxygen species-dependent signaling regulates cancer**. *Cellular and molecular life sciences : CMLS* 2009, **66**(23):3663-3673.

24. Gaude E, Frezza C: **Defects in mitochondrial metabolism and cancer**. *Cancer & metabolism* 2014, **2**:10.

25. Masson N, Ratcliffe PJ: **Hypoxia signaling pathways in cancer metabolism: the importance of co-selecting interconnected physiological pathways**. *Cancer & metabolism* 2014, **2**(1):3.

26. Gano LB, Patel M, Rho JM: **Ketogenic diets, mitochondria, and neurological diseases**. *Journal of lipid research* 2014, **55**(11):2211-2228.

27. Elia M, Klepper J, Leiendecker B, Hartmann H: **Ketogenic Diets in the Treatment of Epilepsy**. *Current pharmaceutical design* 2017, **23**(37):5691-5701.

28. Stafstrom CE, Rho JM: **The ketogenic diet as a treatment paradigm for diverse neurological disorders**. *Frontiers in pharmacology* 2012, **3**:59.

29. Maalouf M, Rho JM, Mattson MP: **The neuroprotective properties of calorie restriction, the ketogenic diet, and ketone bodies**. *Brain research reviews* 2009, **59**(2):293-315.

30. Boison D: **New insights into the mechanisms of the ketogenic diet**. *Current opinion in neurology* 2017, **30**(2):187-192.

31. Cahill GF, Jr., Veech RL: **Ketoacids? Good medicine?** *Transactions of the American Clinical and Climatological Association* 2003, **114**:149-161; discussion 162-143.

32. Morris AA: **Cerebral ketone body metabolism**. *Journal of inherited metabolic disease* 2005, **28**(2):109-121.

33. VanItallie TB, Nufert TH: **Ketones: metabolism's ugly duckling**. *Nutrition reviews* 2003, **61**(10):327-341.

34. Veech RL, Chance B, Kashiwaya Y, Lardy HA, Cahill GF, Jr.: **Ketone bodies, potential therapeutic uses**. *IUBMB life* 2001, **51**(4):241-247.

35. Gasior M, Rogawski MA, Hartman AL: **Neuroprotective and disease-modifying effects of the ketogenic diet**. *Behavioural pharmacology* 2006, **17**(5-6):431-439.

36. Seyfried TN, Kiebish MA, Marsh J, Shelton LM, Huysentruyt LC, Mukherjee P: **Metabolic management of brain cancer**. *Biochimica et biophysica acta* 2011, **1807**(6):577-594.

37. Maurer GD, Brucker DP, Bahr O, Harter PN, Hattingen E, Walenta S, Mueller-Klieser W, Steinbach JP, Rieger J: **Differential utilization of ketone bodies by neurons and glioma cell lines: a rationale for ketogenic diet as experimental glioma therapy**. *BMC cancer* 2011, **11**:315.

38. Zhou W, Mukherjee P, Kiebish MA, Markis WT, Mantis JG, Seyfried TN: **The calorically restricted ketogenic diet, an effective alternative therapy for malignant brain cancer**. *Nutrition & metabolism* 2007, **4**:5.

39. Seyfried TN, Mukherjee P: **Targeting energy metabolism in brain cancer: review and hypothesis**. *Nutrition & metabolism* 2005, **2**:30.

40. Tisdale MJ, Brennan RA: **Loss of acetoacetate coenzyme A transferase activity in tumours of peripheral tissues**. *British journal of cancer* 1983, **47**(2):293-297.

41. Fredericks M, Ramsey RB: **3-Oxo acid coenzyme A transferase activity in brain and tumors of the nervous system**. *Journal of neurochemistry* 1978, **31**(6):1529-1531.

42. Seyfried TN, Marsh J, Shelton LM, Huysentruyt LC, Mukherjee P: **Is the restricted ketogenic diet a viable alternative to the standard of care for managing malignant brain cancer?** *Epilepsy research* 2012, **100**(3):310-326.

43. Seyfried TN, Flores R, Poff AM, D'Agostino DP, Mukherjee P: **Metabolic therapy: a new paradigm for managing malignant brain cancer**. *Cancer letters* 2015, **356**(2 Pt A):289-300.

44. Maroon JC, Seyfried TN, Donohue JP, Bost J: **The role of metabolic therapy in treating glioblastoma multiforme**. *Surgical neurology international* 2015, **6**:61.

45. Bozzetti F, Zupec-Kania B: **Toward a cancer-specific diet**. *Clinical nutrition (Edinburgh, Scotland)* 2016, **35**(5):1188-1195.

46. Seyfried BT, Kiebish M, Marsh J, Mukherjee P: **Targeting energy metabolism in brain cancer through calorie restriction and the ketogenic diet**. *Journal of cancer research and therapeutics* 2009, **5 Suppl 1**:S7-15.

47. Wallace DC, Fan W, Procaccio V: **Mitochondrial energetics and therapeutics**. *Annual review of pathology* 2010, **5**:297-348.

48. Simone BA, Champ CE, Rosenberg AL, Berger AC, Monti DA, Dicker AP, Simone NL: **Selectively starving cancer cells through dietary manipulation: methods and clinical implications**. *Future oncology (London, England)* 2013, **9**(7):959-976.

49. Klement RJ: **Calorie or carbohydrate restriction? The ketogenic diet as another option for supportive cancer treatment**. *The oncologist* 2013, **18**(9):1056.

50. Scheck AC, Abdelwahab MG, Fenton KE, Stafford P: **The ketogenic diet for the treatment of glioma: insights from genetic profiling**. *Epilepsy research* 2012, **100**(3):327-337.

51. Magee BA, Potezny N, Rofe AM, Conyers RA: **The inhibition of malignant cell growth by ketone bodies**. *The Australian journal of experimental biology and medical science* 1979, **57**(5):529-539.

52. Skinner R, Trujillo A, Ma X, Beierle EA: **Ketone bodies inhibit the viability of human neuroblastoma cells**. *Journal of pediatric surgery* 2009, **44**(1):212-216; discussion 216.

53. Shimazu T, Hirschey MD, Newman J, He W, Shirakawa K, Le Moan N, Grueter CA, Lim H, Saunders LR, Stevens RD *et al*: **Suppression of oxidative stress by beta-hydroxybutyrate, an endogenous histone deacetylase inhibitor**. *Science (New York, NY)* 2013, **339**(6116):211-214.

54. Newman JC, Verdin E: **beta-hydroxybutyrate: much more than a metabolite**. *Diabetes research and clinical practice* 2014, **106**(2):173-181.

55. Newman JC, Verdin E: **Ketone bodies as signaling metabolites**. *Trends in endocrinology and metabolism: TEM* 2014, **25**(1):42-52.

56. Sassone-Corsi P: **Physiology. When metabolism and epigenetics converge**. *Science (New York, NY)* 2013, **339**(6116):148-150.

57. Shelton LM, Huysentruyt LC, Mukherjee P, Seyfried TN: **Calorie restriction as an anti-invasive therapy for malignant brain cancer in the VM mouse**. *ASN neuro* 2010, **2**(3):e00038.

58. Marsh J, Mukherjee P, Seyfried TN: **Drug/diet synergy for managing malignant astrocytoma in mice: 2-deoxy-D-glucose and the restricted ketogenic diet**. *Nutrition & metabolism* 2008, **5**:33.

59. Poff AM, Ari C, Arnold P, Seyfried TN, D'Agostino DP: **Ketone supplementation decreases tumor cell viability and prolongs survival of mice with metastatic cancer**. *International journal of cancer* 2014, **135**(7):1711-1720.

60. Poff AM, Ward N, Seyfried TN, Arnold P, D'Agostino DP: **Non-Toxic Metabolic Management of Metastatic Cancer in VM Mice: Novel Combination of Ketogenic Diet, Ketone Supplementation, and Hyperbaric Oxygen Therapy**. *PloS one* 2015, **10**(6):e0127407.

61. Stafford P, Abdelwahab MG, Kim DY, Preul MC, Rho JM, Scheck AC: **The ketogenic diet reverses gene expression patterns and reduces reactive oxygen species levels when used as an adjuvant therapy for glioma**. *Nutr Metab (Lond)* 2010, **7**:74.

62. Woolf EC, Curley KL, Liu Q, Turner GH, Charlton JA, Preul MC, Scheck AC: **The Ketogenic Diet Alters the Hypoxic Response and Affects Expression of Proteins Associated with Angiogenesis, Invasive Potential and Vascular Permeability in a Mouse Glioma Model**. *PLoS ONE* 2015, **10**(6):e0130357.

63. Poff AM, Ari C, Seyfried TN, D'Agostino DP: **The ketogenic diet and hyperbaric oxygen therapy prolong survival in mice with systemic metastatic cancer**. *PloS one* 2013, **8**(6):e65522.

64. Mavropoulos JC, Buschemeyer WC, 3rd, Tewari AK, Rokhfeld D, Pollak M, Zhao Y, Febbo PG, Cohen P, Hwang D, Devi G *et al*: **The effects of varying dietary carbohydrate and fat content on survival in a murine LNCaP prostate cancer xenograft model**. *Cancer prevention research (Philadelphia, Pa)* 2009, **2**(6):557-565.

65. Otto C, Kaemmerer U, Illert B, Muehling B, Pfetzer N, Wittig R, Voelker HU, Thiede A, Coy JF: **Growth of human gastric cancer cells in nude mice is**

delayed by a ketogenic diet supplemented with omega-3 fatty acids and medium-chain triglycerides. *BMC cancer* 2008, **8**:122.

66. Caso J, Masko EM, Ii JA, Poulton SH, Dewhirst M, Pizzo SV, Freedland SJ: **The effect of carbohydrate restriction on prostate cancer tumor growth in a castrate mouse xenograft model**. *The Prostate* 2013, **73**(5):449-454.

67. Freedland SJ, Mavropoulos J, Wang A, Darshan M, Demark-Wahnefried W, Aronson WJ, Cohen P, Hwang D, Peterson B, Fields T *et al*: **Carbohydrate restriction, prostate cancer growth, and the insulin-like growth factor axis**. *The Prostate* 2008, **68**(1):11-19.

68. Hao GW, Chen YS, He DM, Wang HY, Wu GH, Zhang B: **Growth of human colon cancer cells in nude mice is delayed by ketogenic diet with or without omega-3 fatty acids and medium-chain triglycerides**. *Asian Pacific journal of cancer prevention : APJCP* 2015, **16**(5):2061-2068.

69. Kim HS, Masko EM, Poulton SL, Kennedy KM, Pizzo SV, Dewhirst MW, Freedland SJ: **Carbohydrate restriction and lactate transporter inhibition in a mouse xenograft model of human prostate cancer**. *BJU international* 2012, **110**(7):1062-1069.

70. Lv M, Zhu X, Wang H, Wang F, Guan W: **Roles of caloric restriction, ketogenic diet and intermittent fasting during initiation, progression and metastasis of cancer in animal models: a systematic review and meta-analysis**. *PloS one* 2014, **9**(12):e115147.

71. !!! INVALID CITATION !!! {}.

72. Allen BG, Bhatia SK, Buatti JM, Brandt KE, Lindholm KE, Button AM, Szweda LI, Smith BJ, Spitz DR, Fath MA: **Ketogenic diets enhance oxidative stress and radio-chemo-therapy responses in lung cancer xenografts**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2013, **19**(14):3905-3913.

73. Milder J, Patel M: **Modulation of oxidative stress and mitochondrial function by the ketogenic diet**. *Epilepsy research* 2012, **100**(3):295-303.

74. Puchowicz MA, Zechel JL, Valerio J, Emancipator DS, Xu K, Pundik S, LaManna JC, Lust WD: **Neuroprotection in diet-induced ketotic rat brain after focal ischemia**. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* 2008, **28**(12):1907-1916.

75. Kim DY, Hao J, Liu R, Turner G, Shi FD, Rho JM: **Inflammation-mediated memory dysfunction and effects of a ketogenic diet in a murine model of multiple sclerosis**. *PloS one* 2012, **7**(5):e35476.

76. Amann T, Hellerbrand C: **GLUT1 as a therapeutic target in hepatocellular carcinoma**. *Expert opinion on therapeutic targets* 2009, **13**(12):1411-1427.

77. Gluschnaider U, Hertz R, Ohayon S, Smeir E, Smets M, Pikarsky E, Bar-Tana J: **Long-chain fatty acid analogues suppress breast tumorigenesis and progression**. *Cancer Res* 2014, **74**(23):6991-7002.

78. Allen BG, Bhatia SK, Anderson CM, Eichenberger-Gilmore JM, Sibenaller ZA, Mapuskar KA, Schoenfeld JD, Buatti JM, Spitz DR, Fath MA: **Ketogenic diets as an adjuvant cancer therapy: History and potential mechanism**. *Redox biology* 2014, **2**:963-970.

79. Woolf EC, Syed N, Scheck AC: **Tumor Metabolism, the Ketogenic Diet and beta-Hydroxybutyrate: Novel Approaches to Adjuvant Brain Tumor Therapy**. *Frontiers in molecular neuroscience* 2016, **9**:122.

80. Abdelwahab MG, Fenton KE, Preul MC, Rho JM, Lynch A, Stafford P, Scheck AC: **The ketogenic diet is an effective adjuvant to radiation therapy for the treatment of malignant glioma**. *PLoS ONE* 2012, **7**(5):e36197.

81. Klement RJ, Champ CE: **Calories, carbohydrates, and cancer therapy with radiation: exploiting the five R's through dietary manipulation**. *Cancer metastasis reviews* 2014, **33**(1):217-229.

82. Raffaghello L, Lee C, Safdie FM, Wei M, Madia F, Bianchi G, Longo VD: **Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy**. *Proceedings of the National Academy of Sciences of the United States of America* 2008, **105**(24):8215-8220.

83. Raffaghello L, Safdie F, Bianchi G, Dorff T, Fontana L, Longo VD: **Fasting and differential chemotherapy protection in patients**. *Cell cycle (Georgetown, Tex)* 2010, **9**(22):4474-4476.

84. Lee C, Safdie FM, Raffaghello L, Wei M, Madia F, Parrella E, Hwang D, Cohen P, Bianchi G, Longo VD: **Reduced levels of IGF-I mediate differential protection of normal and cancer cells in response to fasting and improve chemotherapeutic index**. *Cancer Res* 2010, **70**(4):1564-1572.

85. Lee C, Raffaghello L, Brandhorst S, Safdie FM, Bianchi G, Martin-Montalvo A, Pistoia V, Wei M, Hwang S, Merlino A *et al*: **Fasting cycles retard growth of tumors and sensitize a range of cancer cell types to chemotherapy**. *Science translational medicine* 2012, **4**(124):124ra127.

86. Safdie F, Brandhorst S, Wei M, Wang W, Lee C, Hwang S, Conti PS, Chen TC, Longo VD: **Fasting enhances the response of glioma to chemo- and radiotherapy**. *PloS one* 2012, **7**(9):e44603.

87. Saleh AD, Simone BA, Palazzo J, Savage JE, Sano Y, Dan T, Jin L, Champ CE, Zhao S, Lim M *et al*: **Caloric restriction augments radiation efficacy in breast cancer**. *Cell cycle (Georgetown, Tex)* 2013, **12**(12):1955-1963.

88. Champ CE, Baserga R, Mishra MV, Jin L, Sotgia F, Lisanti MP, Pestell RG, Dicker AP, Simone NL: **Nutrient restriction and radiation therapy for cancer treatment: when less is more**. *The oncologist* 2013, **18**(1):97-103.

89. Champ CE, Palmer JD, Volek JS, Werner-Wasik M, Andrews DW, Evans JJ, Glass J, Kim L, Shi W: **Targeting metabolism with a ketogenic diet during the treatment of glioblastoma multiforme**. *Journal of neuro-oncology* 2014, **117**(1):125-131.

90. Saenko Y, Cieslar-Pobuda A, Skonieczna M, Rzeszowska-Wolny J: **Changes of reactive oxygen and nitrogen species and mitochondrial functioning in human K562 and HL60 cells exposed to ionizing radiation**. *Radiation research* 2013, **180**(4):360-366.

91. Santivasi WL, Xia F: **Ionizing radiation-induced DNA damage, response, and repair**. *Antioxidants & redox signaling* 2014, **21**(2):251-259.

92. Heydari AR, Unnikrishnan A, Lucente LV, Richardson A: **Caloric restriction and genomic stability**. *Nucleic acids research* 2007, **35**(22):7485-7496.

93. Zhang H, Gu C, Yu J, Wang Z, Yuan X, Yang L, Wang J, Jia Y, Liu J, Liu F: **Radiosensitization of glioma cells by TP53-induced glycolysis and apoptosis regulator knockdown is dependent on thioredoxin-1 nuclear translocation**. *Free radical biology & medicine* 2014, **69**:239-248.

94. Choi EJ, Cho BJ, Lee DJ, Hwang YH, Chun SH, Kim HH, Kim IA: **Enhanced cytotoxic effect of radiation and temozolomide in malignant glioma cells: targeting PI3K-AKT-mTOR signaling, HSP90 and histone deacetylases**. *BMC cancer* 2014, **14**:17.

95. Medova M, Aebersold DM, Zimmer Y: **The Molecular Crosstalk between the MET Receptor Tyrosine Kinase and the DNA Damage Response-Biological and Clinical Aspects**. *Cancers* 2013, **6**(1):1-27.

96. Gil del Alcazar CR, Hardebeck MC, Mukherjee B, Tomimatsu N, Gao X, Yan J, Xie XJ, Bachoo R, Li L, Habib AA *et al*: **Inhibition of DNA double-strand break repair by the dual PI3K/mTOR inhibitor NVP-BEZ235 as a strategy for radiosensitization of glioblastoma**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2014, **20**(5):1235-1248.

97. Munshi A, Ramesh R: **Mitogen-activated protein kinases and their role in radiation response**. *Genes & cancer* 2013, **4**(9-10):401-408.

98. Li W, Guo F, Wang P, Hong S, Zhang C: **miR-221/222 confers radioresistance in glioblastoma cells through activating Akt independent of PTEN status**. *Current molecular medicine* 2014, **14**(1):185-195.

99. Sanli T, Steinberg GR, Singh G, Tsakiridis T: **AMP-activated protein kinase (AMPK) beyond metabolism: a novel genomic stress sensor participating in**

the DNA damage response pathway. *Cancer biology & therapy* 2014, **15**(2):156-169.

100. Danhier P, De Saedeleer CJ, Karroum O, De Preter G, Porporato PE, Jordan BF, Gallez B, Sonveaux P: **Optimization of tumor radiotherapy with modulators of cell metabolism: toward clinical applications**. *Seminars in radiation oncology* 2013, **23**(4):262-272.

101. Wang Y, Yuan JL, Zhang YT, Ma JJ, Xu P, Shi CH, Zhang W, Li YM, Fu Q, Zhu GF *et al*: **Inhibition of both EGFR and IGF1R sensitized prostate cancer cells to radiation by synergistic suppression of DNA homologous recombination repair**. *PloS one* 2013, **8**(8):e68784.

102. Chang HT, Olson LK, Schwartz KA: **Ketolytic and glycolytic enzymatic expression profiles in malignant gliomas: implication for ketogenic diet therapy**. *Nutrition & metabolism* 2013, **10**(1):47.

103. Lund TM, Risa O, Sonnewald U, Schousboe A, Waagepetersen HS: **Availability of neurotransmitter glutamate is diminished when beta-hydroxybutyrate replaces glucose in cultured neurons**. *Journal of neurochemistry* 2009, **110**(1):80-91.

104. Hartman AL: **Neuroprotection in metabolism-based therapy**. *Epilepsy research* 2012, **100**(3):286-294.

105. Fearon KC, Borland W, Preston T, Tisdale MJ, Shenkin A, Calman KC: **Cancer cachexia: influence of systemic ketosis on substrate levels and nitrogen metabolism**. *The American journal of clinical nutrition* 1988, **47**(1):42-48.

106. Nebeling LC, Lerner E: **Implementing a ketogenic diet based on medium-chain triglyceride oil in pediatric patients with cancer**. *Journal of the American Dietetic Association* 1995, **95**(6):693-697.

107. Nebeling LC, Miraldi F, Shurin SB, Lerner E: **Effects of a ketogenic diet on tumor metabolism and nutritional status in pediatric oncology patients: two case reports**. *Journal of the American College of Nutrition* 1995, **14**(2):202-208.

108. Zuccoli G, Marcello N, Pisanello A, Servadei F, Vaccaro S, Mukherjee P, Seyfried TN: **Metabolic management of glioblastoma multiforme using standard therapy together with a restricted ketogenic diet: Case Report**. *Nutrition & metabolism* 2010, **7**:33.

109. Schmidt M, Pfetzer N, Schwab M, Strauss I, Kammerer U: **Effects of a ketogenic diet on the quality of life in 16 patients with advanced cancer: A pilot trial**. *Nutrition & metabolism* 2011, **8**(1):54.

110. Schwartz K, Chang HT, Nikolai M, Pernicone J, Rhee S, Olson K, Kurniali PC, Hord NG, Noel M: **Treatment of glioma patients with ketogenic diets: report of two cases treated with an IRB-approved energy-restricted ketogenic diet protocol and review of the literature**. *Cancer & metabolism* 2015, **3**:3.

111. Tan-Shalaby JL, Carrick J, Edinger K, Genovese D, Liman AD, Passero VA, Shah RB: **Modified Atkins diet in advanced malignancies - final results of a safety and feasibility trial within the Veterans Affairs Pittsburgh Healthcare System**. *Nutrition & metabolism* 2016, **13**:52.

112. Dirven L, Taphoorn MJ, Reijneveld JC, Blazeby J, Jacobs M, Pusic A, La Sala E, Stupp R, Fayers P, Efficace F: **The level of patient-reported outcome reporting in randomised controlled trials of brain tumour patients: a systematic review**. *European journal of cancer (Oxford, England : 1990)* 2014, **50**(14):2432-2448.

113. Boele FW, Heimans JJ, Aaronson NK, Taphoorn MJ, Postma TJ, Reijneveld JC, Klein M: **Health-related quality of life of significant others of patients with malignant CNS versus non-CNS tumors: a comparative study**. *Journal of neuro-oncology* 2013, **115**(1):87-94.

114. van den Bent MJ, Wefel JS, Schiff D, Taphoorn MJ, Jaeckle K, Junck L, Armstrong T, Choucair A, Waldman AD, Gorlia T *et al*: **Response assessment in neuro-oncology (a report of the RANO group): assessment of outcome in trials of diffuse low-grade gliomas**. *The Lancet Oncology* 2011, **12**(6):583-593.

115. Kmiecik J, Poli A, Brons NH, Waha A, Eide GE, Enger PO, Zimmer J, Chekenya M: **Elevated CD3+ and CD8+ tumor-infiltrating immune cells correlate with prolonged survival in glioblastoma patients despite integrated immunosuppressive mechanisms in the tumor microenvironment and at the systemic level**. *J Neuroimmunol* 2013, **264**(1-2):71-83.

116. Razavi SM, Lee KE, Jin BE, Aujla PS, Gholamin S, Li G: **Immune Evasion Strategies of Glioblastoma**. *Frontiers in surgery* 2016, **3**:11.

117. Desai R, Suryadevara CM, Batich KA, Farber SH, Sanchez-Perez L, Sampson JH: **Emerging immunotherapies for glioblastoma**. *Expert opinion on emerging drugs* 2016, **21**(2):133-145.

118. Wintterle S, Schreiner B, Mitsdoerffer M, Schneider D, Chen L, Meyermann R, Weller M, Wiendl H: **Expression of the B7-related molecule B7-H1 by glioma cells: a potential mechanism of immune paralysis**. *Cancer Res* 2003, **63**(21):7462-7467.

119. Berghoff AS, Kiesel B, Widhalm G, Rajky O, Ricken G, Wohrer A, Dieckmann K, Filipits M, Brandstetter A, Weller M *et al*: **Programmed death ligand 1 expression and tumor-infiltrating lymphocytes in glioblastoma**. *Neuro-oncology* 2015, **17**(8):1064-1075.

120. Parsa AT, Waldron JS, Panner A, Crane CA, Parney IF, Barry JJ, Cachola KE, Murray JC, Tihan T, Jensen MC *et al*: **Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma**. *Nat Med* 2007, **13**(1):84-88.

121. Avril T, Saikali S, Vauleon E, Jary A, Hamlat A, De TM, Mosser J, Quillien V: **Distinct effects of human glioblastoma immunoregulatory molecules programmed cell death ligand-1 (PDL-1) and indoleamine 2,3-dioxygenase (IDO) on tumour-specific T cell functions**. *J Neuroimmunol* 2010, **225**(1-2):22-33.

122. Bloch O, Crane CA, Kaur R, Safaee M, Rutkowski MJ, Parsa AT: **Gliomas promote immunosuppression through induction of B7-H1 expression in tumor-associated macrophages**. *Clin Cancer Res* 2013, **19**(12):3165-3175.

123. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K *et al*: **Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion**. *Nat Med* 2002, **8**(8):793-800.

124. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ: **Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses**. *Immunity* 2007, **27**(1):111-122.

125. Xue S, Hu M, Iyer V, Yu J: **Blocking the PD-1/PD-L1 pathway in glioma: a potential new treatment strategy**. *Journal of hematology & oncology* 2017, **10**(1):81.

126. Huang B, Zhang H, Gu L, Ye B, Jian Z, Stary C, Xiong X: **Advances in Immunotherapy for Glioblastoma Multiforme**. *Journal of immunology research* 2017, **2017**:3597613.

127. Lussier DM, Woolf EC, Johnson JL, Brooks KS, Blattman JN, Scheck AC: **Enhanced immunity in a mouse model of malignant glioma is mediated by a therapeutic ketogenic diet**. *BMC cancer* 2016, **16**:310.

128. Weller M, Cloughesy T, Perry JR, Wick W: **Standards of care for treatment of recurrent glioblastoma--are we there yet?** *Neuro-oncology* 2013, **15**(1):4-27.

129. Olar A, Aldape KD: **Using the molecular classification of glioblastoma to inform personalized treatment**. *The Journal of pathology* 2014, **232**(2):165-177.

130. Wei J, Wu A, Kong LY, Wang Y, Fuller G, Fokt I, Melillo G, Priebe W, Heimberger AB: **Hypoxia potentiates glioma-mediated immunosuppression**. *PloS one* 2011, **6**(1):e16195.

131. Joon Yun A, Bazar KA, Lee PY: **Tumors may modulate host immunity partly through hypoxia-induced sympathetic bias**. *Medical hypotheses* 2004, **63**(2):352-356.

132. Bruzzese L, Fromonot J, By Y, Durand-Gorde JM, Condo J, Kipson N, Guieu R, Fenouillet E, Ruf J: **NF-kappaB enhances hypoxia-driven T-cell immunosuppression via upregulation of adenosine A(2A) receptors**. *Cellular signalling* 2014, **26**(5):1060-1067.

133. Ampie L, Woolf EC, Dardis C: **Immunotherapeutic advancements for glioblastoma**. *Frontiers in oncology* 2015, **5**:12.

134. Nduom EK, Weller M, Heimberger AB: **Immunosuppressive mechanisms in glioblastoma**. *Neuro-oncology* 2015, **17 Suppl 7**:vii9-vii14.

135. Wing K, Sakaguchi S: **Regulatory T cells exert checks and balances on self tolerance and autoimmunity**. *Nature immunology* 2010, **11**(1):7-13.

136. Fecci PE, Mitchell DA, Whitesides JF, Xie W, Friedman AH, Archer GE, Herndon JE, 2nd, Bigner DD, Dranoff G, Sampson JH: **Increased regulatory T-cell fraction amidst a diminished CD4 compartment explains cellular immune defects in patients with malignant glioma**. *Cancer Res* 2006, **66**(6):3294-3302.

137. Husain Z, Huang Y, Seth P, Sukhatme VP: **Tumor-derived lactate modifies antitumor immune response: effect on myeloid-derived suppressor cells and NK cells**. *Journal of immunology (Baltimore, Md : 1950)* 2013, **191**(3):1486-1495.

138. Fecci PE, Ochiai H, Mitchell DA, Grossi PM, Sweeney AE, Archer GE, Cummings T, Allison JP, Bigner DD, Sampson JH: **Systemic CTLA-4 blockade ameliorates glioma-induced changes to the CD4+ T cell compartment without affecting regulatory T-cell function**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2007, **13**(7):2158-2167.

139. Vom Berg J, Vrohlings M, Haller S, Haimovici A, Kulig P, Sledzinska A, Weller M, Becher B: **Intratumoral IL-12 combined with CTLA-4 blockade elicits T cell-mediated glioma rejection**. *The Journal of experimental medicine* 2013, **210**(13):2803-2811.

140. Zeng J, See AP, Phallen J, Jackson CM, Belcaid Z, Ruzevick J, Durham N, Meyer C, Harris TJ, Albesiano E *et al*: **Anti-PD-1 blockade and stereotactic radiation produce long-term survival in mice with intracranial gliomas**. *International journal of radiation oncology, biology, physics* 2013, **86**(2):343-349.

141. Wainwright DA, Chang AL, Dey M, Balyasnikova IV, Kim CK, Tobias A, Cheng Y, Kim JW, Qiao J, Zhang L *et al*: **Durable therapeutic efficacy utilizing combinatorial blockade against IDO, CTLA-4, and PD-L1 in mice with brain tumors**. *Clin Cancer Res* 2014, **20**(20):5290-5301.

142. Reardon DA, Gokhale PC, Klein SR, Ligon KL, Rodig SJ, Ramkissoon SH, Jones KL, Conway AS, Liao X, Zhou J *et al*: **Glioblastoma Eradication Following Immune Checkpoint Blockade in an Orthotopic, Immunocompetent Model**. *Cancer Immunol Res* 2016, **4**(2):124-135.

143. Platten M, Reardon DA: **Concepts for Immunotherapies in Gliomas**. *Seminars in neurology* 2018, **38**(1):62-72.

144. Yu P, Fu YX: **Tumor-infiltrating T lymphocytes: friends or foes?** *Laboratory investigation; a journal of technical methods and pathology* 2006, **86**(3):231-245.

145. Hagemann T, Wilson J, Burke F, Kulbe H, Li NF, Pluddemann A, Charles K, Gordon S, Balkwill FR: **Ovarian cancer cells polarize macrophages toward a tumor-associated phenotype**. *Journal of immunology (Baltimore, Md : 1950)* 2006, **176**(8):5023-5032.

146. Banchereau J, Steinman RM: **Dendritic cells and the control of immunity**. *Nature* 1998, **392**(6673):245-252.

147. Schroder K, Hertzog PJ, Ravasi T, Hume DA: **Interferon-gamma: an overview of signals, mechanisms and functions**. *Journal of leukocyte biology* 2004, **75**(2):163-189.

148. Muller-Hubenthal B, Azemar M, Lorenzen D, Huber M, Freudenberg MA, Galanos C, Unger C, Hildenbrand B: **Tumour Biology: tumour-associated inflammation versus antitumor immunity**. *Anticancer research* 2009, **29**(11):4795-4805.

149. Grivennikov SI, Greten FR, Karin M: **Immunity, inflammation, and cancer**. *Cell* 2010, **140**(6):883-899.

150. Kohrt HE, Nouri N, Nowels K, Johnson D, Holmes S, Lee PP: **Profile of immune cells in axillary lymph nodes predicts disease-free survival in breast cancer**. *PLoS medicine* 2005, **2**(9):e284.

151. Procaccini C, Galgani M, De Rosa V, Matarese G: **Intracellular metabolic pathways control immune tolerance**. *Trends in immunology* 2012, **33**(1):1-7.

152. Rathmell JC, Vander Heiden MG, Harris MH, Frauwirth KA, Thompson CB: **In the absence of extrinsic signals, nutrient utilization by lymphocytes is insufficient to maintain either cell size or viability**. *Molecular cell* 2000, **6**(3):683-692.

153. Frauwirth KA, Thompson CB: **Regulation of T lymphocyte metabolism**. *Journal of immunology (Baltimore, Md : 1950)* 2004, **172**(8):4661-4665.

154. Yap M, Brouard S, Pecqueur C, Degauque N: **Targeting CD8 T-Cell Metabolism in Transplantation**. *Frontiers in immunology* 2015, **6**:547.

155. Lochner M, Berod L, Sparwasser T: **Fatty acid metabolism in the regulation of T cell function**. *Trends in immunology* 2015, **36**(2):81-91.

156. Molon B, Cali B, Viola A: **T Cells and Cancer: How Metabolism Shapes Immunity**. *Frontiers in immunology* 2016, **7**:20.

157. Sukumar M, Roychoudhuri R, Restifo NP: **Nutrient Competition: A New Axis of Tumor Immunosuppression**. *Cell* 2015, **162**(6):1206-1208.

158. Abdelwahab MG, Sankar T, Preul MC, Scheck AC: **Intracranial implantation with subsequent 3D in vivo bioluminescent imaging of murine gliomas**. *Journal of visualized experiments : JoVE* 2011(57):e3403.

159. Jouanneau E, Poujol D, Gulia S, Le Mercier I, Blay JY, Belin MF, Puisieux I: **Dendritic cells are essential for priming but inefficient for boosting antitumour immune response in an orthotopic murine glioma model**. *Cancer immunology, immunotherapy : CII* 2006, **55**(3):254-267.

160. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, Ahmed R: **Viral immune evasion due to persistence of activated T cells without effector function**. *The Journal of experimental medicine* 1998, **188**(12):2205-2213.

161. Villarete L, Somasundaram T, Ahmed R: **Tissue-mediated selection of viral variants: correlation between glycoprotein mutation and growth in neuronal cells**. *Journal of virology* 1994, **68**(11):7490-7496.

162. de Andrade PV, Andrade AF, de Paula Queiroz RG, Scrideli CA, Tone LG, Valera ET: **The histone deacetylase inhibitor PCI-24781 as a putative radiosensitizer in pediatric glioblastoma cell lines**. *Cancer cell international* 2016, **16**:31.

163. Rivera S, Leteur C, Megnin F, Law F, Martins I, Kloos I, Depil S, Modjtahedi N, Perfettini JL, Hennequin C *et al*: **Time dependent modulation of tumor radiosensitivity by a pan HDAC inhibitor: abexinostat**. *Oncotarget* 2017, **8**(34):56210-56227.

164. Shi W, Palmer JD, Werner-Wasik M, Andrews DW, Evans JJ, Glass J, Kim L, Bar-Ad V, Judy K, Farrell C *et al*: **Phase I trial of panobinostat and fractionated stereotactic re-irradiation therapy for recurrent high grade gliomas**. *Journal of neuro-oncology* 2016, **127**(3):535-539.

165. Watanabe S, Kuwabara Y, Suehiro S, Yamashita D, Tanaka M, Tanaka A, Ohue S, Araki H: **Valproic acid reduces hair loss and improves survival in patients receiving temozolomide-based radiation therapy for high-grade glioma**. *European journal of clinical pharmacology* 2017, **73**(3):357-363.

166. Pilones KA, Vanpouille-Box C, Demaria S: **Combination of radiotherapy and immune checkpoint inhibitors**. *Seminars in radiation oncology* 2015, **25**(1):28-33.

167. Sharabi AB, Lim M, DeWeese TL, Drake CG: **Radiation and checkpoint blockade immunotherapy: radiosensitisation and potential mechanisms of synergy**. *The Lancet Oncology* 2015, **16**(13):e498-509.

168. Twyman-Saint Victor C, Rech AJ, Maity A, Rengan R, Pauken KE, Stelekati E, Benci JL, Xu B, Dada H, Odorizzi PM *et al*: **Radiation and dual checkpoint**

blockade activate non-redundant immune mechanisms in cancer. *Nature* 2015, **520**(7547):373-377.

169. Lee JH, Choy ML, Ngo L, Foster SS, Marks PA: **Histone deacetylase inhibitor induces DNA damage, which normal but not transformed cells can repair**. *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**(33):14639-14644.

170. Groselj B, Sharma NL, Hamdy FC, Kerr M, Kiltie AE: **Histone deacetylase inhibitors as radiosensitisers: effects on DNA damage signalling and repair**. *British journal of cancer* 2013, **108**(4):748-754.

171. Stengel KR, Hiebert SW: **Class I HDACs Affect DNA Replication, Repair, and Chromatin Structure: Implications for Cancer Therapy**. *Antioxidants & redox signaling* 2015, **23**(1):51-65.

172. Iliakis GE, Okayasu R: **Radiosensitivity throughout the cell cycle and repair of potentially lethal damage and DNA double-strand breaks in an X-ray-sensitive CHO mutant**. *International journal of radiation biology* 1990, **57**(6):1195-1211.

173. Di Cerbo V, Schneider R: **Cancers with wrong HATs: the impact of acetylation**. *Briefings in functional genomics* 2013, **12**(3):231-243.

174. Li Y, Seto E: **HDACs and HDAC Inhibitors in Cancer Development and Therapy**. *Cold Spring Harbor perspectives in medicine* 2016, **6**(10).

175. Staberg M, Michaelsen SR, Rasmussen RD, Villingshoj M, Poulsen HS, Hamerlik P: **Inhibition of histone deacetylases sensitizes glioblastoma cells to lomustine**. *Cellular oncology (Dordrecht)* 2017, **40**(1):21-32.

176. Rasmussen RD, Gajjar MK, Jensen KE, Hamerlik P: **Enhanced efficacy of combined HDAC and PARP targeting in glioblastoma**. *Molecular oncology* 2016, **10**(5):751-763.

177. Shimazu T, Hirschey MD, Newman J, He W, Shirakawa K, Le Moan N, Grueter CA, Lim H, Saunders LR, Stevens RD *et al*: **Suppression of oxidative stress by beta-hydroxybutyrate, an endogenous histone deacetylase inhibitor**. *Science* 2013, **339**(6116):211-214.

178. Lee DH, Ryu HW, Won HR, Kwon SH: **Advances in epigenetic glioblastoma therapy**. *Oncotarget* 2017, **8**(11):18577-18589.

179. Erasimus H, Gobin M, Niclou S, Van Dyck E: **DNA repair mechanisms and their clinical impact in glioblastoma**. *Mutation research Reviews in mutation research* 2016, **769**:19-35.

180. Xia SJ, Shammas MA, Shmookler Reis RJ: **Elevated recombination in immortal human cells is mediated by HsRAD51 recombinase**. *Molecular and cellular biology* 1997, **17**(12):7151-7158.

181. Raderschall E, Stout K, Freier S, Suckow V, Schweiger S, Haaf T: **Elevated levels of Rad51 recombination protein in tumor cells**. *Cancer Res* 2002, **62**(1):219-225.

182. Hannay JA, Liu J, Zhu QS, Bolshakov SV, Li L, Pisters PW, Lazar AJ, Yu D, Pollock RE, Lev D: **Rad51 overexpression contributes to chemoresistance in human soft tissue sarcoma cells: a role for p53/activator protein 2 transcriptional regulation**. *Molecular cancer therapeutics* 2007, **6**(5):1650-1660.

183. Collis SJ, Tighe A, Scott SD, Roberts SA, Hendry JH, Margison GP: **Ribozyme minigene-mediated RAD51 down-regulation increases radiosensitivity of human prostate cancer cells**. *Nucleic acids research* 2001, **29**(7):1534-1538.

184. Hine CM, Seluanov A, Gorbunova V: **Use of the Rad51 promoter for targeted anti-cancer therapy**. *Proceedings of the National Academy of Sciences of the United States of America* 2008, **105**(52):20810-20815.

185. Ohnishi T, Taki T, Hiraga S, Arita N, Morita T: **In vitro and in vivo potentiation of radiosensitivity of malignant gliomas by antisense inhibition of the RAD51 gene**. *Biochemical and biophysical research communications* 1998, **245**(2):319-324.

186. Saydam O, Saydam N, Glauser DL, Pruschy M, Dinh-Van V, Hilbe M, Jacobs AH, Ackermann M, Fraefel C: **HSV-1 amplicon-mediated post-transcriptional inhibition of Rad51 sensitizes human glioma cells to ionizing radiation**. *Gene therapy* 2007, **14**(15):1143-1151.

187. Quiros S, Roos WP, Kaina B: **Rad51 and BRCA2--New molecular targets for sensitizing glioma cells to alkylating anticancer drugs**. *PloS one* 2011, **6**(11):e27183.

188. Welsh JW, Mahadevan D, Ellsworth R, Cooke L, Bearss D, Stea B: **The c-Met receptor tyrosine kinase inhibitor MP470 radiosensitizes glioblastoma cells**. *Radiation oncology (London, England)* 2009, **4**:69.

189. King HO, Brend T, Payne HL, Wright A, Ward TA, Patel K, Egnuni T, Stead LF, Patel A, Wurdak H *et al*: **RAD51 Is a Selective DNA Repair Target to Radiosensitize Glioma Stem Cells**. *Stem cell reports* 2017, **8**(1):125-139.

190. Balbous A, Cortes U, Guilloteau K, Rivet P, Pinel B, Duchesne M, Godet J, Boissonnade O, Wager M, Bensadoun RJ *et al*: **A radiosensitizing effect of RAD51 inhibition in glioblastoma stem-like cells**. *BMC cancer* 2016, **16**:604.

191. Short SC, Giampieri S, Worku M, Alcaide-German M, Sioftanos G, Bourne S, Lio KI, Shaked-Rabi M, Martindale C: **Rad51 inhibition is an effective means of targeting DNA repair in glioma models and CD133+ tumor-derived cells**. *Neuro-oncology* 2011, **13**(5):487-499.

192. Han X, Xue X, Zhou H, Zhang G: **A molecular view of the radioresistance of gliomas**. *Oncotarget* 2017, **8**(59):100931-100941.

193. Silva-Nichols HB, Woolf EC, Deleyrolle LP, Reynolds BA, Scheck AC: **ATPS-77THE KETONE BODY ß-HYDROXYBUTYRATE RADIOSENSITIZES GLIOBLASTOMA MULTIFORME STEM CELLS**. *Neuro-oncology* 2015, **17**(Suppl 5):v35.

194. Wu Z, Jing S, Li Y, Gao Y, Yu S, Li Z, Zhao Y, Piao J, Ma S, Chen X: **The effects of SAHA on radiosensitivity in pancreatic cancer cells by inducing apoptosis and targeting RAD51**. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* 2017, **89**:705-710.

195. Krumm A, Barckhausen C, Kucuk P, Tomaszowski KH, Loquai C, Fahrer J, Kramer OH, Kaina B, Roos WP: **Enhanced Histone Deacetylase Activity in Malignant Melanoma Provokes RAD51 and FANCD2-Triggered Drug Resistance**. *Cancer Res* 2016, **76**(10):3067-3077.

196. Lai TH, Ewald B, Zecevic A, Liu C, Sulda M, Papaioannou D, Garzon R, Blachly JS, Plunkett W, Sampath D: **HDAC Inhibition Induces MicroRNA-182, which Targets RAD51 and Impairs HR Repair to Sensitize Cells to Sapacitabine in Acute Myelogenous Leukemia**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2016, **22**(14):3537-3549.

197. Shoji M, Ninomiya I, Makino I, Kinoshita J, Nakamura K, Oyama K, Nakagawara H, Fujita H, Tajima H, Takamura H *et al*: **Valproic acid, a histone deacetylase inhibitor, enhances radiosensitivity in esophageal squamous cell carcinoma**. *International journal of oncology* 2012, **40**(6):2140-2146.

198. Adimoolam S, Sirisawad M, Chen J, Thiemann P, Ford JM, Buggy JJ: **HDAC inhibitor PCI-24781 decreases RAD51 expression and inhibits homologous recombination**. *Proceedings of the National Academy of Sciences of the United States of America* 2007, **104**(49):19482-19487.

199. Staedtke V, Bai RY, Laterra J: **Investigational new drugs for brain cancer**. *Expert Opin Investig Drugs* 2016, **25**(8):937-956.

200. Chen R, Cohen AL, Colman H: **Targeted Therapeutics in Patients With High-Grade Gliomas: Past, Present, and Future**. *Current treatment options in oncology* 2016, **17**(8):42.

201. Qiu X, Xiao X, Li N, Li Y: **Histone deacetylases inhibitors (HDACis) as novel therapeutic application in various clinical diseases**. *Progress in neuro-psychopharmacology & biological psychiatry* 2017, **72**:60-72.

202. Hornig E, Heppt MV, Graf SA, Ruzicka T, Berking C: **Inhibition of histone deacetylases in melanoma-a perspective from bench to bedside**. *Experimental dermatology* 2016, **25**(11):831-838.

203. Horing E, Podlech O, Silkenstedt B, Rota IA, Adamopoulou E, Naumann U: **The histone deacetylase inhibitor trichostatin a promotes apoptosis and antitumor immunity in glioblastoma cells**. *Anticancer research* 2013, **33**(4):1351-1360.

204. Terranova-Barberio M, Thomas S, Ali N, Pawlowska N, Park J, Krings G, Rosenblum MD, Budillon A, Munster PN: **HDAC inhibition potentiates immunotherapy in triple negative breast cancer**. *Oncotarget* 2017, **8**(69):114156-114172.

205. Zheng H, Zhao W, Yan C, Watson CC, Massengill M, Xie M, Massengill C, Noyes DR, Martinez GV, Afzal R *et al*: **HDAC Inhibitors Enhance T-Cell Chemokine Expression and Augment Response to PD-1 Immunotherapy in Lung Adenocarcinoma**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2016, **22**(16):4119-4132.

206. Mazzone R, Zwergel C, Mai A, Valente S: **Epi-drugs in combination with immunotherapy: a new avenue to improve anticancer efficacy**. *Clinical epigenetics* 2017, **9**:59.

207. Cacan E: **Epigenetic-mediated immune suppression of positive co-stimulatory molecules in chemoresistant ovarian cancer cells**. *Cell biology international* 2017, **41**(3):328-339.

208. Woods DM, Sodre AL, Villagra A, Sarnaik A, Sotomayor EM, Weber J: **HDAC Inhibition Upregulates PD-1 Ligands in Melanoma and Augments Immunotherapy with PD-1 Blockade**. *Cancer immunology research* 2015, **3**(12):1375-1385.

209. Jaworski DM, Namboodiri AM, Moffett JR: **Acetate as a Metabolic and Epigenetic Modifier of Cancer Therapy**. *Journal of cellular biochemistry* 2016, **117**(3):574-588.

210. Youm YH, Nguyen KY, Grant RW, Goldberg EL, Bodogai M, Kim D, D'Agostino D, Planavsky N, Lupfer C, Kanneganti TD *et al*: **The ketone metabolite beta-hydroxybutyrate blocks NLRP3 inflammasome-mediated inflammatory disease**. *Nature medicine* 2015, **21**(3):263-269.

211. Veech RL: **Ketone ester effects on metabolism and transcription**. *Journal of lipid research* 2014, **55**(10):2004-2006.

212. Veech RL: **The therapeutic implications of ketone bodies: the effects of ketone bodies in pathological conditions: ketosis, ketogenic diet, redox states, insulin resistance, and mitochondrial metabolism**. *Prostaglandins, leukotrienes, and essential fatty acids* 2004, **70**(3):309-319.

213. Kashiwaya Y, Bergman C, Lee JH, Wan R, King MT, Mughal MR, Okun E, Clarke K, Mattson MP, Veech RL: **A ketone ester diet exhibits anxiolytic and cognition-sparing properties, and lessens amyloid and tau pathologies in a**

mouse model of Alzheimer's disease. *Neurobiology of aging* 2013, **34**(6):1530-1539.

214. Shukla SK, Gebregiworgis T, Purohit V, Chaika NV, Gunda V, Radhakrishnan P, Mehla K, Pipinos, II, Powers R, Yu F *et al*: **Metabolic reprogramming induced by ketone bodies diminishes pancreatic cancer cachexia**. *Cancer & metabolism* 2014, **2**:18.

215. Newport MT, VanItallie TB, Kashiwaya Y, King MT, Veech RL: **A new way to produce hyperketonemia: use of ketone ester in a case of Alzheimer's disease**. *Alzheimer's & dementia : the journal of the Alzheimer's Association* 2015, **11**(1):99-103.

216. Crane C, Panner A, Pieper RO, Arbiser J, Parsa AT: **Honokiol-mediated inhibition of PI3K/mTOR pathway: a potential strategy to overcome immunoresistance in glioma, breast, and prostate carcinoma without impacting T cell function**. *Journal of immunotherapy (Hagerstown, Md : 1997)* 2009, **32**(6):585-592.

217. McDaniel SS, Rensing NR, Thio LL, Yamada KA, Wong M: **The ketogenic diet inhibits the mammalian target of rapamycin (mTOR) pathway**. *Epilepsia* 2011, **52**(3):e7-11.

218. Martuscello RT, Vedam-Mai V, McCarthy DJ, Schmoll ME, Jundi MA, Louviere CD, Griffith BG, Skinner CL, Suslov O, Deleyrolle LP *et al*: **A Supplemented High-Fat Low-Carbohydrate Diet for the Treatment of Glioblastoma**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2016, **22**(10):2482-2495.

219. Gowrishankar K, Gunatilake D, Gallagher SJ, Tiffen J, Rizos H, Hersey P: **Inducible but not constitutive expression of PD-L1 in human melanoma cells is dependent on activation of NF-kappaB**. *PloS one* 2015, **10**(4):e0123410.

220. Fu SP, Li SN, Wang JF, Li Y, Xie SS, Xue WJ, Liu HM, Huang BX, Lv QK, Lei LC *et al*: **BHBA suppresses LPS-induced inflammation in BV-2 cells by inhibiting NF-kappaB activation**. *Mediators of inflammation* 2014, **2014**:983401.

221. Bally AP, Austin JW, Boss JM: **Genetic and Epigenetic Regulation of PD-1 Expression**. *Journal of immunology (Baltimore, Md : 1950)* 2016, **196**(6):2431-2437.

222. Staron MM, Gray SM, Marshall HD, Parish IA, Chen JH, Perry CJ, Cui G, Li MO, Kaech SM: **The transcription factor FoxO1 sustains expression of the inhibitory receptor PD-1 and survival of antiviral CD8(+) T cells during chronic infection**. *Immunity* 2014, **41**(5):802-814.

223. Kao C, Oestreich KJ, Paley MA, Crawford A, Angelosanto JM, Ali MA, Intlekofer AM, Boss JM, Reiner SL, Weinmann AS *et al*: **Transcription factor T-bet**

represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection. *Nature immunology* 2011, **12**(7):663-671.

224. Crane CA, Ahn BJ, Han SJ, Parsa AT: **Soluble factors secreted by glioblastoma cell lines facilitate recruitment, survival, and expansion of regulatory T cells: implications for immunotherapy**. *Neuro-oncology* 2012, **14**(5):584-595.

225. Cahill KE, Morshed RA, Yamini B: **Nuclear factor-kappaB in glioblastoma: insights into regulators and targeted therapy**. *Neuro-oncology* 2016, **18**(3):329-339.

226. Husain Z, Seth P, Sukhatme VP: **Tumor-derived lactate and myeloid-derived suppressor cells: Linking metabolism to cancer immunology**. *Oncoimmunology* 2013, **2**(11):e26383.

227. Hayes J, Peruzzi PP, Lawler S: **MicroRNAs in cancer: biomarkers, functions and therapy**. *Trends in molecular medicine* 2014, **20**(8):460-469.

228. Melo SA, Moutinho C, Ropero S, Calin GA, Rossi S, Spizzo R, Fernandez AF, Davalos V, Villanueva A, Montoya G *et al*: **A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells**. *Cancer cell* 2010, **18**(4):303-315.

229. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA *et al*: **MicroRNA expression profiles classify human cancers**. *Nature* 2005, **435**(7043):834-838.

230. Li Q, Liu Q: **Noncoding RNAs in Cancer Immunology**. *Advances in experimental medicine and biology* 2016, **927**:243-264.

231. Wang Q, Lin W, Tang X, Li S, Guo L, Lin Y, Kwok HF: **The Roles of microRNAs in Regulating the Expression of PD-1/PD-L1 Immune Checkpoint**. *International journal of molecular sciences* 2017, **18**(12).

232. Ali SR, Humphreys KJ, McKinnon RA, Michael MZ: **Impact of Histone Deacetylase Inhibitors on microRNA Expression and Cancer Therapy: A Review**. *Drug development research* 2015, **76**(6):296-317.

233. Shea A, Harish V, Afzal Z, Chijioke J, Kedir H, Dusmatova S, Roy A, Ramalinga M, Harris B, Blancato J *et al*: **MicroRNAs in glioblastoma multiforme pathogenesis and therapeutics**. *Cancer medicine* 2016, **5**(8):1917-1946.

234. Huang SW, Ali ND, Zhong L, Shi J: **MicroRNAs as biomarkers for human glioblastoma: progress and potential**. *Acta pharmacologica Sinica* 2018.

235. Wang Y, Wang L: **miR-34a attenuates glioma cells progression and chemoresistance via targeting PD-L1**. *Biotechnology letters* 2017, **39**(10):1485-1492.

236. Cortez MA, Ivan C, Valdecanas D, Wang X, Peltier HJ, Ye Y, Araujo L, Carbone DP, Shilo K, Giri DK *et al*: **PDL1 Regulation by p53 via miR-34**. *Journal of the National Cancer Institute* 2016, **108**(1).

237. Wang X, Li J, Dong K, Lin F, Long M, Ouyang Y, Wei J, Chen X, Weng Y, He T *et al*: **Tumor suppressor miR-34a targets PD-L1 and functions as a potential immunotherapeutic target in acute myeloid leukemia**. *Cellular signalling* 2015, **27**(3):443-452.

238. Balzeau J, Menezes MR, Cao S, Hagan JP: **The LIN28/let-7 Pathway in Cancer**. *Frontiers in genetics* 2017, **8**:31.

239. Liu X, Jiang L, Wang A, Yu J, Shi F, Zhou X: **MicroRNA-138 suppresses invasion and promotes apoptosis in head and neck squamous cell carcinoma cell lines**. *Cancer letters* 2009, **286**(2):217-222.

240. Yeh YM, Chuang CM, Chao KC, Wang LH: **MicroRNA-138 suppresses ovarian cancer cell invasion and metastasis by targeting SOX4 and HIF-1alpha**. *International journal of cancer* 2013, **133**(4):867-878.

241. Qiu S, Huang D, Yin D, Li F, Li X, Kung HF, Peng Y: **Suppression of tumorigenicity by microRNA-138 through inhibition of EZH2-CDK4/6-pRb-E2F1 signal loop in glioblastoma multiforme**. *Biochimica et biophysica acta* 2013, **1832**(10):1697-1707.

242. Yu Z, Wang Z, Li F, Yang J, Tang L: **miR138 modulates prostate cancer cell invasion and migration via Wnt/betacatenin pathway**. *Molecular medicine reports* 2018, **17**(2):3140-3145.

243. Zhang Q, He Y, Nie M, Cai W: **Roles of miR-138 and ISG15 in oral squamous cell carcinoma**. *Experimental and therapeutic medicine* 2017, **14**(3):2329-2334.

244. Sha HH, Wang DD, Chen D, Liu SW, Wang Z, Yan DL, Dong SC, Feng JF: **MiR-138: A promising therapeutic target for cancer**. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* 2017, **39**(4):1010428317697575.

245. Zhao L, Yu H, Yi S, Peng X, Su P, Xiao Z, Liu R, Tang A, Li X, Liu F *et al*: **The tumor suppressor miR-138-5p targets PD-L1 in colorectal cancer**. *Oncotarget* 2016, **7**(29):45370-45384.

246. Berindan-Neagoe I, Calin GA: **Molecular pathways: microRNAs, cancer cells, and microenvironment**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2014, **20**(24):6247-6253.

247. Wei J, Nduom EK, Kong LY, Hashimoto Y, Xu S, Gabrusiewicz K, Ling X, Huang N, Qiao W, Zhou S *et al*: **MiR-138 exerts anti-glioma efficacy by targeting immune checkpoints**. *Neuro-oncology* 2016, **18**(5):639-648.

248. Wei J, Wang F, Kong LY, Xu S, Doucette T, Ferguson SD, Yang Y, McEnery K, Jethwa K, Gjyshi O *et al*: **miR-124 inhibits STAT3 signaling to enhance T cell-mediated immune clearance of glioma**. *Cancer Res* 2013, **73**(13):3913-3926.

249. Peng Y, Croce CM: **The role of MicroRNAs in human cancer**. *Signal transduction and targeted therapy* 2016, **1**:15004.

250. Van Roosbroeck K, Calin GA: **Cancer Hallmarks and MicroRNAs: The Therapeutic Connection**. *Advances in cancer research* 2017, **135**:119-149.

251. Chen L, Kang C: **miRNA interventions serve as 'magic bullets' in the reversal of glioblastoma hallmarks**. *Oncotarget* 2015, **6**(36):38628-38642.

252. Dalmay T, Edwards DR: **MicroRNAs and the hallmarks of cancer**. *Oncogene* 2006, **25**(46):6170-6175.

253. Vannini I, Fanini F, Fabbri M: **Emerging roles of microRNAs in cancer**. *Current opinion in genetics & development* 2018, **48**:128-133.

254. Bertoli G, Cava C, Castiglioni I: **MicroRNAs: New Biomarkers for Diagnosis, Prognosis, Therapy Prediction and Therapeutic Tools for Breast Cancer**. *Theranostics* 2015, **5**(10):1122-1143.

255. Cortez MA, Valdecanas D, Niknam S, Peltier HJ, Diao L, Giri U, Komaki R, Calin GA, Gomez DR, Chang JY *et al*: **In Vivo Delivery of miR-34a Sensitizes Lung Tumors to Radiation Through RAD51 Regulation**. *Molecular therapy Nucleic acids* 2015, **4**:e270.

256. Betel D, Koppal A, Agius P, Sander C, Leslie C: **Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites**. *Genome biology* 2010, **11**(8):R90.

257. Betel D, Wilson M, Gabow A, Marks DS, Sander C: **The microRNA.org resource: targets and expression**. *Nucleic acids research* 2008, **36**(Database issue):D149-153.

258. John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS: **Human MicroRNA targets**. *PLoS biology* 2004, **2**(11):e363.

259. Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS: **MicroRNA targets in Drosophila**. *Genome biology* 2003, **5**(1):R1.

260. Agarwal V, Bell GW, Nam JW, Bartel DP: **Predicting effective microRNA target sites in mammalian mRNAs**. *eLife* 2015, **4**.

261. Miranda KC, Huynh T, Tay Y, Ang YS, Tam WL, Thomson AM, Lim B, Rigoutsos I: **A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes**. *Cell* 2006, **126**(6):1203-1217.

262. Ostrom QT, Gittleman H, Xu J, Kromer C, Wolinsky Y, Kruchko C, Barnholtz-Sloan JS: **CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2009-2013**. *Neuro-oncology* 2016, **18**(suppl_5):v1-v75.

263. Zhang X, Zhu S, Li T, Liu YJ, Chen W, Chen J: **Targeting immune checkpoints in malignant glioma**. *Oncotarget* 2017, **8**(4):7157-7174.

264. Huang RY, Francois A, McGray AR, Miliotto A, Odunsi K: **Compensatory upregulation of PD-1, LAG-3, and CTLA-4 limits the efficacy of single-agent checkpoint blockade in metastatic ovarian cancer**. *Oncoimmunology* 2017, **6**(1):e1249561.

265. Zhai L, Lauing KL, Chang AL, Dey M, Qian J, Cheng Y, Lesniak MS, Wainwright DA: **The role of IDO in brain tumor immunotherapy**. *Journal of neuro-oncology* 2015, **123**(3):395-403.

266. Hanihara M, Kawataki T, Oh-Oka K, Mitsuka K, Nakao A, Kinouchi H: **Synergistic antitumor effect with indoleamine 2,3-dioxygenase inhibition and temozolomide in a murine glioma model**. *Journal of neurosurgery* 2016, **124**(6):1594-1601.

267. Lan J, Li R, Yin LM, Deng L, Gui J, Chen BQ, Zhou L, Meng MB, Huang QR, Mo XM *et al*: **Targeting Myeloid-derived Suppressor Cells and Programmed Death Ligand 1 Confers Therapeutic Advantage of Ablative Hypofractionated Radiation Therapy Compared With Conventional Fractionated Radiation Therapy**. *International journal of radiation oncology, biology, physics* 2018, **101**(1):74-87.

268. Pitroda SP, Stack ME, Liu GF, Song SS, Chen L, Liang H, Parekh AD, Huang X, Roach P, Posner MC *et al*: **JAK2 Inhibitor SAR302503 Abrogates PD-L1 Expression and Targets Therapy-Resistant Non-small Cell Lung Cancers**. *Molecular cancer therapeutics* 2018, **17**(4):732-739.

269. Skinner HD, Giri U, Yang LP, Kumar M, Liu Y, Story MD, Pickering CR, Byers LA, Williams MD, Wang J *et al*: **Integrative Analysis Identifies a Novel AXL-PI3 Kinase-PD-L1 Signaling Axis Associated with Radiation Resistance in Head and Neck Cancer**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2017, **23**(11):2713-2722.

270. Oweida A, Lennon S, Calame D, Korpela S, Bhatia S, Sharma J, Graham C, Binder D, Serkova N, Raben D *et al*: **Ionizing radiation sensitizes tumors to PD-L1 immune checkpoint blockade in orthotopic murine head and neck squamous cell carcinoma**. *Oncoimmunology* 2017, **6**(10):e1356153.

271. Morisada M, Clavijo PE, Moore E, Sun L, Chamberlin M, Van Waes C, Hodge JW, Mitchell JB, Friedman J, Allen CT: **PD-1 blockade reverses adaptive immune resistance induced by high-dose hypofractionated but not low-dose daily fractionated radiation**. *Oncoimmunology* 2018, **7**(3):e1395996.

272. Lhuillier C, Vanpouille-Box C, Galluzzi L, Formenti SC, Demaria S: **Emerging biomarkers for the combination of radiotherapy and immune checkpoint blockers**. *Seminars in cancer biology* 2017.