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Glutamine metabolism fuels de novo purine synthesis in Multiple Myeloma for DNA replication and cell proliferation

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An abstract of
a thesis submitted to the Faculty of
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Abstract

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Multiple Myeloma is the second most common hematological malignancy and accounts for approximately 10% of all blood cancers. It is currently considered incurable which stresses the need for better therapies. Myeloma cells have shown an interesting dependence on glutamine uptake and upregulation of glutamine transporters. Genetic perturbation of the glutamine transporter ASCT2 has been shown to reduce tumor growth in myeloma and small molecule inhibitors targeting ASCT2, such as V-9302, have had anti-tumor effects in several cancers in preclinical studies. The current literature in myeloma suggests that glutamine is needed by myeloma cells to replenish the TCA cycle intermediate alpha ketoglutarate and sustain ATP synthesis. This hypothesis is based on the high activity of the glutaminase enzyme GLS1 which hydrolyzes glutamine to glutamate. However, there are several metabolic pathways that involve glutamine and play key roles in myeloma including glutathione synthesis, protein glycosylation, and nucleotide synthesis. Our bioinformatics analysis suggested that de novo purine synthesis was an important pathway for glutamine in myeloma and could be playing a key role in glutamine addiction seen in myeloma.

My hypothesis was that myeloma cells increase glutamine uptake and glutamine dependence after transformation because of the need to synthesize purines *de novo* to fuel DNA replication for increased proliferation. Rewired metabolism is an established feature of cancer in order to meet the metabolic needs of a cancer cell. Plasma cells are terminally differentiated non-dividing cells which means that new pathways must be turned on to sustain cell growth and division when they transform into myeloma cells. Our findings show a cytostatic effect of glutamine deprivation on myeloma cells. Similarly, when we knocked out genes in the de novo purine synthesis pathway using CRISPR/Cas9 we saw a reduction in proliferation, signifying that this is a key pathway for myeloma growth. Overall, our findings suggest that targeting de novo purine synthesis and glutamine metabolism could be an effective myeloma therapy.

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What is Multiple Myeloma?

Multiple myeloma (MM) is a malignancy of plasma cells, the antibody-secreting cells of the immune system. Plasma cells are the terminally differentiated state of B lymphocytes and are capable of secreting 10^3 antibodies per second (Khodadadi et al., 2019). They reside in the bone marrow and can live anywhere from several months to a person's entire lifetime. The onset of different oncogenic events can lead to the transformation of plasma cells to cancerous myeloma cells.

Staging and Progression of Multiple Myeloma

The progression of multiple myeloma is classified into four different stages. The first stage, monoclonal gammopathy of undetermined significance (MGUS), is considered a premalignant stage in which only about 1% of patients per year progress to Multiple Myeloma (Rajkumar and Kumar, 2016). The second stage is Smoldering Myeloma which is more advanced than MGUS although patients remain asymptomatic. There are several subgroups within the overarching Smoldering Myeloma group with some being designated "high risk" and some "low risk" (Rajkumar et al., 2015). The third stage, Multiple Myeloma, is the first symptomatic stage of the disease. It is characterized by the CRAB features which stands for hyper**c**alcemia, **r**enal failure, **a**nemia, and **b**one lesions. Multiple Myeloma patients can present with only one bone marrow lesion however; the disease gets the name Multiple Myeloma because approximately 90% of patients present with more than one lesion (cancer.net). Plasma cell leukemia is the most aggressive stage which occurs when the disease becomes rapidly proliferative and is no longer confined to the bone marrow (Gundesen et al., 2019). Patients with plasma cell leukemia have an average overall survival of less than one year (Gonsalves et al.,

2014). There is another plasma cell malignancy that does not fit in with the other stages of myeloma because it is not a systemic disease called a solitary plasmacytoma. Solitary plasmacytomas can either form in the bone as bone plasmacytoma or in soft tissue referred to as an extramedullary plasmacytoma (Grammatico et al., 2017).

Current Treatments for Multiple Myeloma

Multiple myeloma is currently considered incurable, however many options for treatment are available. The most common treatments for multiple myeloma include proteasome inhibitors, immunomodulatory agents, corticosteroids, autologous stem cell transplant, chemotherapy, and targeted immunotherapies (Rajkumar and Kumar, 2020). These treatments are typically given in a variety of combinations. Currently no metabolism targeting treatments for myeloma are in the clinic however studies performed in vitro and in mice targeting metabolic pathways have shown promise (El Arfani et al., 2018).

One of the biggest successes in myeloma therapy has been the use of proteasome inhibitors. Proteasome inhibitors such as bortezomib or carfilzomib target the plasma cell biology of myeloma cells by taking advantage of their predisposition to synthesize tens of thousands of antibodies per second. The massive amount of translation occurring in myeloma cells requires activity of the proteasome to degrade misfolded protein. Inhibition of the proteasome leads to a terminal unfolded protein response and ultimately apoptosis (Obeng et al., 2006). A common frontline treatment for patients ineligible for autologous stem cell transplant is

a combination of proteasome inhibition with a corticosteroid and an immunomodulatory drug (ImID).

Recent developments in immunotherapy have improved the outcomes for myeloma patients. CD38 is a cell surface marker that is expressed on plasma cells and a few other subtypes of immune cells. The immunotherapy agent daratumumab is a monoclonal antibody that targets CD38. Daratumumab was first approved by the FDA in 2015 as a single agent for patients who had become refractory to other therapies. Since then, it has gained approval in a much wider variety of settings and is now approved as a frontline therapy in combination with previously used frontline therapies (Durie et al., 2020).

Another development in immunotherapy for multiple myeloma is Chimeric Antigen Receptor (CAR) T cells targeting BCMA. A CAR-T cell is an engineered T cell that is programed to target cancer cells. A patient's immune cells will be taken via a blood draw and the T cells will be isolated and transduced to express a receptor that specifically targets an antigen on the cancer cell. BCMA is a cell surface antigen that is expressed only on mature B lymphocytes and myeloma cells. This makes it an attractive target for CAR-T cells because it minimizes the off target effects that a less specific antigen would have. BCMA targeting CAR-T cells are currently in clinical trials. Antibody drug conjugates (ADC) targeting BCMA for myeloma therapy have also had success. GSK received FDA approval in 2020 for their ADC belantamab mafodotin for relapsed or refractory myeloma.

Glutamine Metabolism

Glutamine is a nonessential amino acid that becomes conditionally essential in multiple myeloma. Glutamine metabolism has been studied in myeloma however further investigation is needed to completely understand how myeloma cells metabolize glutamine. The three transporters primarily involved in glutamine transport are SLC1A5 (ASCT2), SLC38A1 (SNAT1), and SLC7A5 (LAT1). Of these three glutamine transporters, only inhibition of ASCT2 reduced the glutamine uptake and growth of myeloma cells (Bolzoni et al., 2016). It has been shown that SNAT1 is upregulated in response to the deletion of ASCT2 in HeLa cells to maintain glutamine uptake. In these cells, ASCT2 deletion did not result in a net decrease in glutamine transport because increased SNAT1 was able to compensate for the loss of ASCT2 (Bröer et al., 2016). However, deletion and inhibition of ASCT2 in myeloma cells has been shown to decrease cell viability and net glutamine uptake. The LAT1 transporter is an antiporter that uptakes leucine in exchange for glutamine. Leucine is an important amino acid for cancer cells because it is involved in activating mTOR for cell growth and proliferation (Drummond and Rasmussen, 2008).

Previous work from the Shanmugam lab found that a majority of myeloma cell lines and patient samples that underwent glutamine and glucose deprivation separately were more sensitive to glutamine deprivation than glucose deprivation (Bajpai et al., 2016). This finding indicates the importance of glutamine and the pathways in which it is metabolized, and provides rationale for targeting these pathways therapeutically.

Glutamine synthetase (GS) is downregulated in myeloma and glutaminase (GLS1) is upregulated (Bolzoni et al., 2016). GLS1 converts glutamine into glutamate and one molecule of ammonia. Glutamate can then be converted into the anapleurotic substrate alpha-ketoglutarate by glutamate dehydrogenase. High GLS1 activity indicates that glutamine is being used to fuel the forward TCA cycle through its conversion into alpha ketoglutarate. GS uses a glutamate and ammonia molecule to make glutamine. Because this study found that GS activity is very low it suggests that the cells glutamine addiction is occurring because of the need to sustain glutamate synthesis.

Glutamine and glutathione synthesis

Glutamate alternatively can be used to synthesize glutathione (GSH). Glutathione plays an important role in antioxidant defense, storage of cysteine, cell cycle progression, apoptosis, redox homeostasis, and modulation of immune function. It is composed of a glutamate and cysteine amino acid bonded together at the gamma carboxyl group of the glutamate and the amino terminus of the cysteine. The amino acid transporter SLC7A11 is an antiporter than exchanges an intracellular glutamate for an extracellular cystine. Cystine is the oxidized dimer of cysteine and can be converted to two cysteine molecules inside the cell (Lu, 2013). Thus glutamate is doubly important for glutathione synthesis because it is involved in the transport of cysteine and is used in the reaction to make glutathione.

Glutathione is used in antioxidant defense through the enzyme GSH-peroxidase (GPx). The reaction catalyzed by GPx reduces hydrogen peroxide and lipid peroxides and oxidizes GSH to its oxidized form GSSG. GSSH is then reduced back to GSH in a reaction catalyzed by GSSG

reductase, which uses up a molecule of NADPH (Lu, 2013). Thus glutathione plays a key role in defending cells against oxidative stress.

Cancer cells typically experience more oxidative stress due to signaling from oncogenes and enhanced metabolism. Multiple myeloma experiences additional oxidative stress due to their continuous secretion of immunoglobulin. Thus decreasing the myeloma cells' ability to handle increased ROS production could be a potential therapeutic avenue. If glutamine deprivation reduces the GSH/GSSG ratio in myeloma cells it could explain why myeloma cells are addicted to glutamine.

Glutathione is also important for redox signaling in the cell. It is capable of reversibly binding to thiol groups on the cysteine residues of proteins in a process called glutathionylation which, similar to phosphorylation, can activate or inactivate the protein. Many important signaling molecules and transcription factors have cysteine residues that can be oxidized and it is one of the ways in which reactive oxygen species regulate cell signaling. Glutathionylation prevents oxidation of these residues and protects them from reactive oxygen species (Lu, 2013). Increased glutathione is associated with cell cycle progression and proliferation in both normal and malignant cells. In a cancer cell that is rapidly proliferative, maintaining glutathione levels is likely very important for proliferation and could explain why glutamine deprivation has a cytostatic effect.

In a study of triple negative breast cancer, a subset of samples was found to be glutamine auxotrophic, meaning they required exogenous glutamine to survive (Timmerman et al., 2013). It

was found that the glutamine auxotrophic cells expressed high levels of the xCT transporter. The xCT transporter (SLC7A11) exports glutamate to take in cystine. Cystine import is critical to the synthesis of glutathione. Inhibition of xCT with sulfasalazine decreased glutathione levels, and also decreased resistance against reactive oxygen species (ROS). This finding implies that glutamine can play an important role in the uptake of cystine, and that the need to defend against ROS in a cancer cell can create glutamine auxotrophy.

Glutathione and Bortezomib Resistance

Glutathione also plays a key role in bortezomib resistance in myeloma. Depletion of glutathione levels has been shown to increase sensitivity of myeloma cells to proteasome inhibition (Starheim et al., 2016). Additionally, bortezomib resistant cells expressed higher levels of the xCT transporter which is essential for maintaining intracellular cysteine levels and for the synthesis of glutathione. Inhibition of the xCT transporter predictably reduced glutathione levels in myeloma cells, and Starheim et al. showed that this also sensitized the cells to bortezomib. This study lays the foundation for glutamine metabolism targeted therapies being used in combination with bortezomib to increase the efficacy of proteasome inhibition.

Glutamine and de novo Purine synthesis

The nitrogen from glutamine plays an important role in nucleotide synthesis. The enzyme phosphoribosyl, pyrophosphate amidotransferase (PPAT) is the first enzyme in the de novo purine synthesis pathway that utilizes glutamine as a substrate.

A study in Small Cell Lung Cancer (SCLC) showed that an increase in the ratio of PPAT/GLS was indicative of tumor progression. Untransformed cells showed a higher flux of glutamine through the anaplerosis pathway whereas malignant cells showed an upregulation of the nucleotide biosynthesis pathway (Kodama et al., 2020). The knockdown of PPAT suppressed the growth of malignant cells. This finding implies that a shift in glutamine metabolism from anaplerosis to de novo nucleotide synthesis could be essential to transformation and cancer growth.

Another study looked at glutamine metabolism in Kaposi's sarcoma associated herpesvirus (KSHV) transformed cells. The transformed cells showed an increase in overall glutamine metabolism, and notably made the cells more dependent on the gamma-nitrogen group of glutamine. These transformed cells upregulated glutaminolysis enzymes such as GLS2, GLUD1, GOT2 indicating an increased reliance of glutamine flux through the TCA cycle. However, when the cells were deprived glutamine, metabolic intermediates such as glutamate, alpha ketoglutarate, and aspartate, were unable to rescue cell viability. When the cells were supplemented with asparagine, which contains a gamma-nitrogen, their viability was rescued. This gamma-nitrogen plays an essential role in de novo purine synthesis and this finding suggests that it is the gamma nitrogen of glutamine that makes these cells sensitive to glutamine deprivation, not the carbons.

Glutamine and Aging

Changes in glutamine metabolism occur during the aging process. In skeletal muscle, glutamine comprises 50-60% of the amino acid pool (Coqueiro et al., 2019). Glutamine plays a key role in mTOR activation which is involved in the regulation of skeletal muscle mass and protein

synthesis. Sarcopenia, the loss of muscle mass that commonly occurs during aging, can be triggered by low glutamine levels (Meynial-Denis, 2016). Studies performed supplementing glutamine and other amino acids such as leucine to reverse sarcopenia have been unsuccessful. It has also been demonstrated in old rats that glutamine synthesis and release are decreased in skeletal muscle cells due to aging.

Glutamine in the Tumor Microenvironment and Effects on Immunogenicity

Competition for resources in the microenvironment of the tumor can lead to the inactivation of immune cells leading to cold tumors. Studies have shown however that glutamine is selectively taken up by cancer cells and that tumor infiltrating myeloid cells preferably use glucose to meet their metabolic needs (Reinfeld et al., 2021). This means that targeting glutamine metabolism could be cancer specific within the tumor microenvironment without negatively affecting immune cells.

Targeting glutamine metabolism has been shown to increase immunogenicity of cancer cells. Depriving cells of glutamine has been shown to increase PD-L1 levels in cancer cells and synergize with PD-1/PD-L1 checkpoint inhibitors (Byun et al., 2020). Inhibition of de novo purine synthesis has been shown to increase response to checkpoint inhibitors in multiple cancer types in in vivo studies (Keshet et al., 2020). This finding could mean that inhibiting glutamine metabolism in multiple myeloma could have an effect on response to immunotherapy because of the connection between glutamine metabolism and de novo purine synthesis. These finding combined with the earlier finding that glutamine is selectively taken up by cancer cells could

mean that therapies targeting glutamine metabolism could improve response to immunotherapy without impacting the function of the immune cells.

Purine Metabolism

Nucleotides are essential to many key cellular processes such as DNA replication, mRNA transcription, tRNA synthesis, and ribosomal formation. There are two classes of nucleotides, purines and pyrimidines. The two purine nucleotides are adenosine and guanosine. Purines can either be synthesized de novo, recycled via the purine salvage pathway or imported through a nucleoside transporter (Cass et al., 2002).

The de novo synthesis pathway consists of nine enzymes. The proximal metabolite for de novo purine synthesis is phosphoribosyl pyrophosphate (PRPP) which comes from the pentose phosphate pathway (PPP) (Hove-Jensen et al., 2017). The initial committed step of de novo purine synthesis is catalyzed by phosphoribosyl pyrophosphate amidotransferase (PPAT) which uses glutamine and PRPP as substrates to make 5-PRA (Goswami et al., 2015). This step is referred to as the initial committed step because there are several pathways through which PRPP can be metabolized and this enzymatic step irreversibly destines PRPP to become a purine nucleotide.

The second step of de novo purine synthesis is catalyzed by glycinamide ribonucleotide transformylase (GART), a trifunctional enzyme composed of three subunits GARS, GARTfase, and AIRS (Batool et al., 2017). GART takes 5-PRA and using glycine and N^{10}-formyl-THF as substrates converts it to phosphoribosyl-N-formylglycinamide.

The third step involves the enzyme phosphoribosylformylglycinamide synthase (PFAS) which, similar to PPAT, uses glutamine as a substrate to convert phosphoribosyl-N-formylglycinamide into N-formylglycinamidine ribonucleotide

GART catalyzes the fourth step of de novo purine synthesis converting N-formylglycinamidine ribonucleotide to aminoimidazole ribotide. Following that step is the conversion of aminoimidazole ribotide to succinylaminoimidazolecarboxamide ribotide by PAICS which is then converted to aminoimidazole carboxamide by ADSL.

ATIC converts aminoimidazole carboxamide to inosine monophosphate (IMP) which is where the de novo purine synthesis pathway branches into making either AMP or GMP.

Glutamine is a substrate in the initial committed step of de novo purine synthesis catalyzed by PPAT, which makes it an essential metabolite for de novo purine synthesis. It is also involved in the third step and it is important for aspartate biosynthesis which is used in fifth step. It is also important to note that all of the carbon and nitrogen in the purine structure comes either directly or indirectly from glutamine, serine, and folate metabolism.

Experimental Model

Because our bioinformatics and metabolomics data implicated de novo purine synthesis as being an essential pathway for glutamine in myeloma, we decided to use CRISPR/CAS9 to directly KO genes in the pathway. The rate limiting enzyme in de novo purine synthesis is PRPP synthetase. However, the metabolite PRPP is not specific to de novo purine synthesis. It is also used in the purine salvage pathway, to make NAD+, and to synthesize the amino acids tryptophan and histidine (Hove Jensen et al.). The first committal step to de novo purine synthesis is catalyzed by the enzyme Phosphoribosyl Pyrophosphate Amidotransferase (PPAT). This enzyme uses PRPP and glutamine as substrates to make the next intermediate in the pathway, PRA. We used CRISPR/CAS9 to knock out PPAT in 4 different myeloma cell lines. Using the Depmap database, created by the Broad Institute at MIT and Harvard, we selected two cell lines that had relatively high dependency on PPAT and two cell lines that had a relatively low dependency on PPAT. Using these knock outs as tool compounds, we sought to characterize the role of PPAT in myeloma cell division, proliferation, DNA replication, and immunogenicity.

Scope of the Thesis

The aim of this thesis is to uncover the basis for elevated glutamine dependency and increased glutamine uptake by myeloma cells. Previous literature in myeloma has tried to explain the addiction of glutamine and the effectiveness of ASCT2 targeted small molecules and genetic perturbations to disrupting glutamine's role in anaplerosis and contribution to ATP synthesis. I believe that this in an incomplete picture of glutamine's role in myeloma growth and progression.

Based on bioinformatics data we hypothesized that glutamine's nitrogens played a key role in de novo purine synthesis. This thesis addresses this hypothesis by (i) showing that ASCT2 inhibition restricts myeloma growth; (ii) replenishing TCA cycle intermediates is not sufficient to restore normal proliferation in glutamine deprived cells; (iii) CRISPR/Cas9 KO of de novo purine synthesis genes restricts tumor growth. Our findings show the importance of de novo purine synthesis in myeloma growth.

Materials and Methods

CRISPR/CAS9 Knock Out:

sgRNA guides were designed using Benchling and confirmed using the Broad Institute database. The guides targeted regions with the most overlap between gene transcripts. The sgRNA guides were then annealed and ligated into the LentiCRISPRv2 plasmid using the restriction enzyme bsmb1. Next plasmids were transformed into E. coli and selected with ampicillin. Then the plasmids were extracted using a DNA miniprep kit and then sent for sequencing. Next HEK 293T cells were transfected with the plasmid and virus was collected after three days of incubation. Finally, the virus from HEK293T cells was used to transduce myeloma cell lines and the KO was confirmed by western blot. The guides used for KO are listed below.

Enzyme	Guide sequence	Forward oligomer	Reverse oligomer
PPAT	ATGAAGTGTTTCAACAACGA exon 3	CACCGATGAAGTGTTTCAACAACGA	AAACTCGTTGTTGAAACACTTCATC
	ATCTTGGAATTGGACACACC exon 3	CACCGATCTTGGAATTGGACACACC	AAACGGTGTGTCCAATTCCAAGATC
	CTGGTATTGTGACTAGTGAT exon 2	CACCGCTGGTATTGTGACTAGTGAT	AAACATCACTAGTCACAATACCAGC

The first two guides targeting exon 3 were successful in knocking out PPAT however, the cells that were transduced with guide 3 were not viable after transduction.

V-9302 Drug sensitivity assay

The sensitivity of KMS11 and L363 to V-9302, an ASCT2 inhibitor, was tested by Cell Titer Glo. 10,000 cells per well were plated in a 96 well plate. V-9302 was added and cells were incubated with inhibitor for 24 hours. After incubation CellTiterGlo reagent was added and fluorescence was read by plate reader.

Western Blot:

Whole cell lysates were made using RIPA buffer. The concentration of the isolated proteins was determined using Biorad Protein Estimation Assay. Thirty micrograms of protein were separated on a 5-20% gradient polyacrylamide gel and transferred to a PVDF membrane. Membranes were then blocked and incubated with primary antibody overnight. Secondary antibody was added next day and membranes were imaged using enhanced chemiluminescence. Beta-actin was used to standardize protein loading.

Proliferation Assay:

125,000 cells/ml were plated in triplicate on a 6 well plate. Cells were counted on days 1, 3, and 5 to assess proliferation. Biorad automatic cell counter was used for cell counts and cells were stained with trypan blue to assess viability.

Results

ASCT2 Expression is Increased in Myeloma and Inhibition of ASCT2 restricts growth

The glutamine transporter ASCT2 has been the subject of previous studies in myeloma and other cancers. It has been shown that knockdown of ASCT2 using shRNA restricts tumor growth in mice. Through an analysis of the GSE6477 dataset we showed that expression of ASCT2 increases across myeloma disease progression. We also tested the drug sensitivity of two myeloma cell lines to V-9302, an inhibitor of ASCT2. We showed that the IC50 of V-9302 in these myeloma cell lines is around 10-11uM by Cell Titer Glo depending on the cell line.

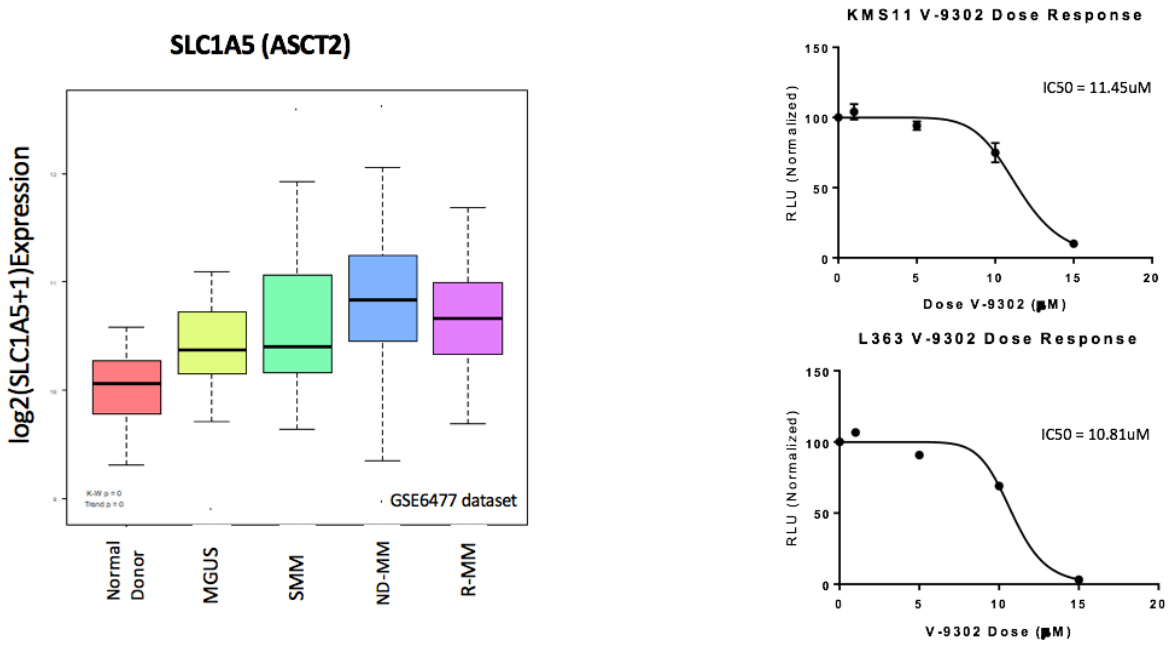

Figure 1: ASCT2 Expression is Increased in Myeloma and Inhibition of ASCT2 restricts growth GSE6477 dataset analysis performed by Emory Bioinformatics shows expression of glutamine transporter ASCT2 is elevated across stages of myeloma. Many myeloma cell lines and patient samples are more sensitive to glutamine deprivation than to glucose deprivation. (A) from GSE6477 dataset. (B) Cell Titer Glo data showing sensitivity of myeloma cell lines KMS11 and L363 to V-9302.

Expression of de novo Purine Synthesis Pathway Upregulated Across Disease Progression

After seeing that expression of genes involved in de novo purine synthesis were highly correlated with ASCT2 expression based on CoMMpass data, we decided to see how the expression of these genes changed across disease progression. We used two different datasets to test this hypothesis. The first dataset we used was the GSE6477 dataset which consists of microarray data from patient samples. The data was separated into 5 groups which included normal donor, MGUS, SMM, ND-MM, and R-MM. We saw that expression of all enzymes in the pathway increased across disease state.

The other dataset we used was from a paper published in Nature Cancer from Irene Ghobrial's lab (Zavidij et al., 2020). We queried this dataset to look at expression of these enzymes across disease progression. Just like in the GSE6477 dataset, we saw that the de novo purine synthesis pathway was upregulated in myeloma compared to healthy bone marrow. These results show that myeloma cells need to turn on de novo purine synthesis after transformation. Healthy plasma cells in the bone marrow do not proliferate. When they become myeloma cells they must become proliferative which entails adjusting metabolic pathways to meet the need for growth.

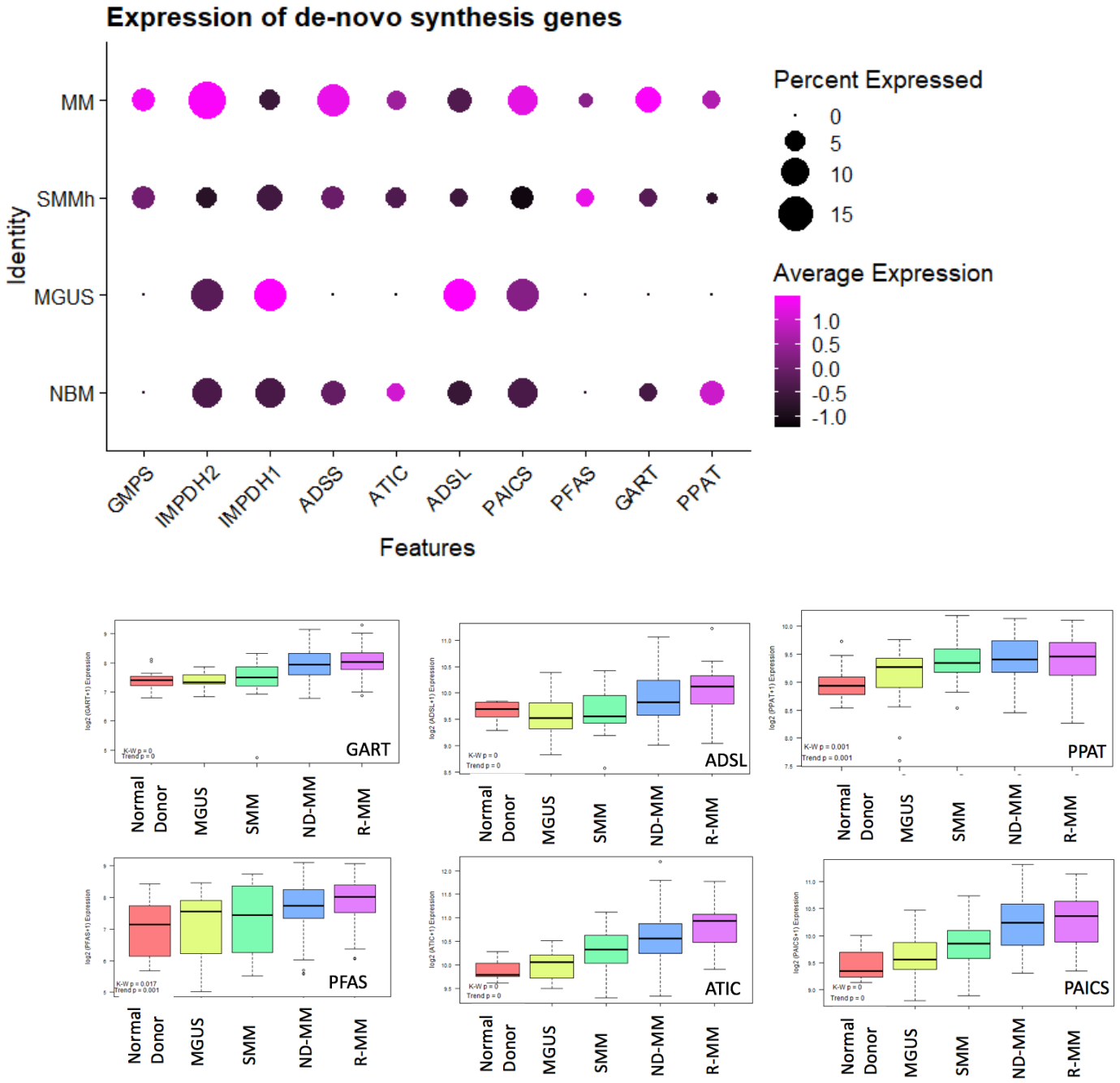

Figure 2: Expression of de novo Purine Synthesis Pathway Upregulated Across Disease Progression. *de novo* purine synthesis pathway is elevated in myeloma. (A) single cell RNA sequencing data from (Zavidij et al., 2020) analyzed by Anjali Mittal, Nagrath Lab University of Michigan. (B) GSE6477 dataset analysis by Emory Bioinformatics showing upregulation of de novo purine synthesis pathway.

PPAT Expression Correlated with Worse Patient OS and PFS

After seeing that the de novo purine synthesis was upregulated in myeloma, we wanted to see the effect that this pathway had on patient survival. Because of the connection that PPAT has to glutamine metabolism and because it is the first step of de novo purine synthesis we decided to analyze the CoMMpass database to see if there is a connection between PPAT Expression and patient survival. We saw that high PPAT was significantly correlated with worse overall survival and progression free survival than low PPAT. This finding indicates that targeting PPAT and de novo purine synthesis could have an impact on patient survival.

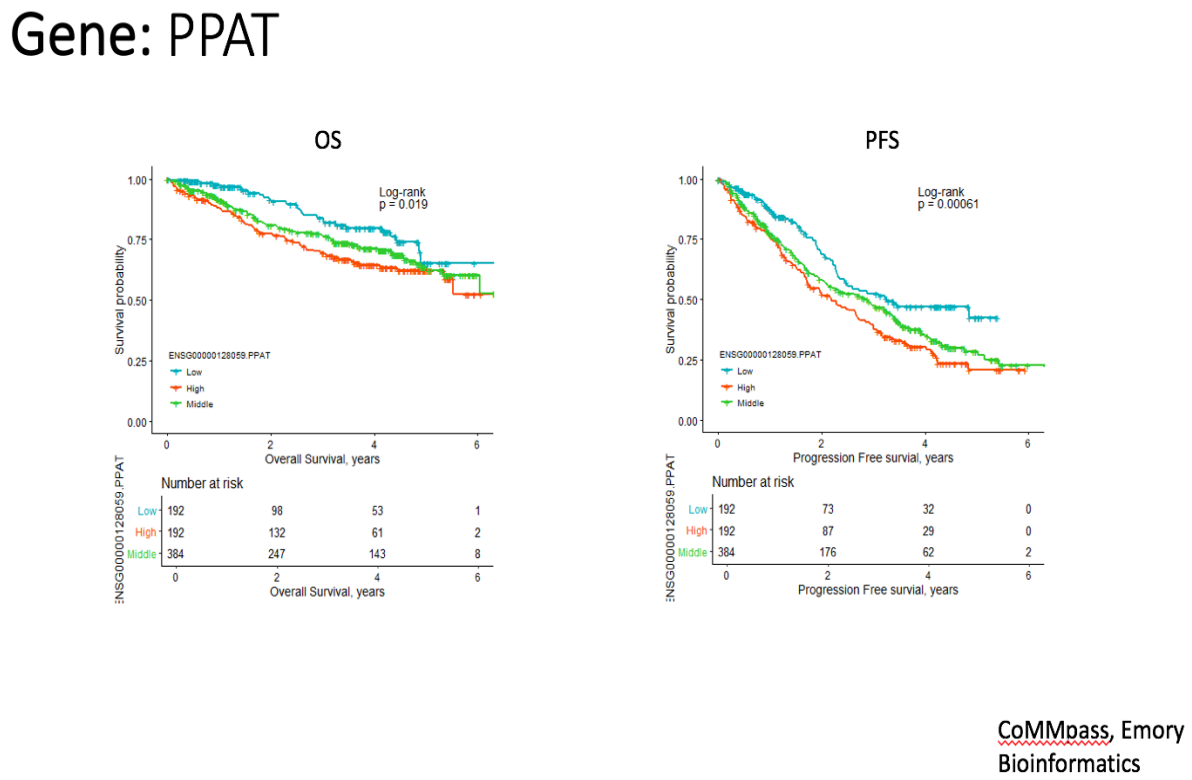

Figure 3: PPAT Expression Correlated with Worse Patient OS and PFS. An analysis of the CoMMpass database version IA15 performed by the Emory Bioinformatics Core. Patients with high PPAT have worse overall survival and progression free survival than patients with low or middle expression.

Metabolite Profiling Data Suggests Glutamine Deprivation Creates Roadblock in de novo Purine Synthesis

Through metabolite profiling data performed on the myeloma cell line L363 we analyzed how different metabolite concentrations changed when deprived of glutamine. When myeloma cells are deprived of glutamine there is an interesting accumulation of PRPP the substrate of PPAT. This indicates that under glutamine deprivation the activity of this enzyme is greatly reduced. A reduction in the activity of PPAT is predicted to shut down the de novo purine synthesis pathway and reduce the available nucleotide pool for proliferation. Glutamine deprivation is also predicted to impact the PFAS and PAICS enzymes. PFAS also uses glutamine directly as a substrate and PAICS uses aspartate as a substrate. The metabolite profiling data shows that aspartate levels are greatly reduced under glutamine deprivation. These metabolite impacts are likely at least partially responsible for the effects we see on cell growth under glutamine deprivation.

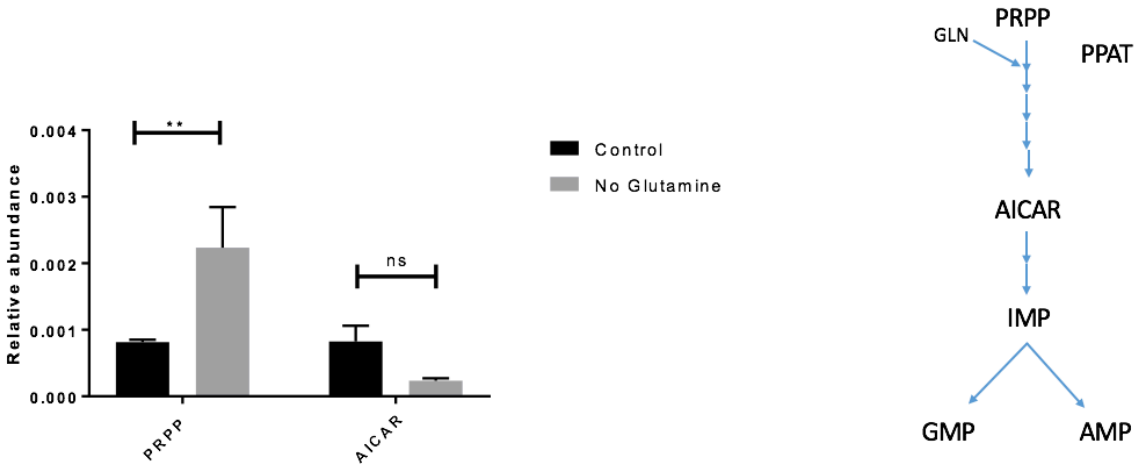

Figure 4: Metabolite Profiling Data Suggests Glutamine Deprivation Creates Roadblock in de novo Purine Synthesis. Glutamine deprivation blocks several key steps in de novo purine synthesis and causes an accumulation of the upstream metabolite PRPP. Metabolite profiling was performed by Human Metabolome Technologies and standardized to internal control.

PPAT KO causes reduced proliferation in myeloma cell line KMS11

Through our bioinformatics analysis we identified PPAT as a target for CRISPR/Cas9 KO. Two of the guides targeting the third exon of PPAT were able to successfully reduce expression of the enzyme. We also saw a reduction of proliferation in the PPAT KO's when compared to the CRISPR control. This finding illustrates PPAT's role in myeloma proliferation. This was excellent validation that de novo purine synthesis plays a key role in myeloma growth and could be a therapeutic target.

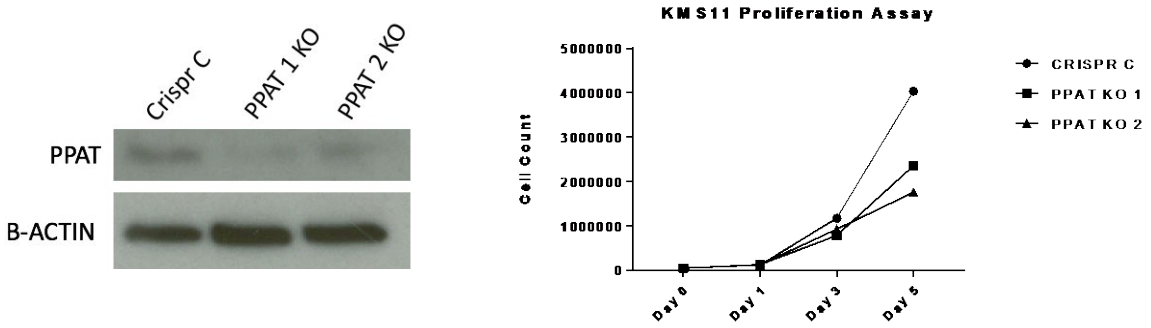

Figure 5: PPAT KO causes reduced proliferation in myeloma cell line KMS11. Using CRISPR/Cas9, PPAT was knocked out in KMS11 and the effect on proliferation was assessed using a cell counting based proliferation assay. The PPAT KO

Discussion

Multiple myeloma treatment has improved dramatically over the last two decades. However, the disease remains incurable which shows the need for innovation in myeloma therapy. Current common treatments such as autologous bone marrow transplant (ASCT) and proteasome inhibition can have serious side effects that greatly impact patient quality of life. Thus the need is twofold for better curative therapies and therapies with fewer side effects.

Cancer metabolism is an emerging field in cancer research that seeks to understand how the metabolic needs of cancer cells differ from healthy cells and how that can be exploited for therapy. This investigation into glutamine metabolism and how it fuels growth and proliferation of myeloma cells sought to improve our understanding of myeloma development and growth. The hope is that it will provide rationale for myeloma therapies targeting glutamine metabolism, perhaps in combination with targeted therapies for de novo purine synthesis, that will improve therapy for myeloma patients.

Our investigation into glutamine metabolism began with a bioinformatics analysis to find genes and pathways that were correlated with increased ASCT2 expression. When we saw that de novo serine synthesis genes were highly upregulated we decided to investigate where the overlap between glutamine and serine metabolism was. De novo purine synthesis is a key overlap between glutamine and serine metabolism so we decided to look at the dependency of those genes in the Broad Institute's Depmap. Using CoMMpass we also looked at how the expression of genes in de novo purine synthesis affected patient survival. We saw that high expression of PPAT was significantly associated with worse prognosis than patients with low PPAT expression. We also used metabolite profiling data from the myeloma cell line L363 to see which metabolites were impacted by glutamine deprivation. We saw that PRPP, the first

metabolite in de novo purine synthesis and a substrate of PPAT was accumulated during glutamine deprivation. This accumulation suggested that the flux through the pathway was shut down during glutamine deprivation and that glutamine was a limiting reagent. Using CRISPR/Cas9 to knock out PPAT, a key enzyme in de novo purine synthesis, we saw that proliferation was reduced in KMS11.

There are several limitations to the study. One of the main limitations is that we were unable to create complete KO lines using CRISPR/Cas9. After transduction with the lentiviral vector containing the CRISPR/Cas9 construct containing a puromycin resistance gene, cells with the KO were selected for using puromycin. After selection with puromycin and a partial KO had been confirmed by western blot, the cells underwent single cell sorting using FACS. However, none of the single cell clones that were screened for expressed a full PPAT KO. We believe that this could be because of the intense selective pressure of single cell cloning. Clones with a PPAT KO would have significantly impaired growth because PPAT is involved in a key growth pathway. In the future using an inducible CRISPR/Cas9 system could improve the efficacy of the KO.

Previous myeloma literature on glutamine metabolism had focused on glutaminolysis and glutamine's role in anaplerosis and ATP synthesis. The research done for this thesis has expanded that view to include the important nitrogen donor role that glutamine plays in de novo purine synthesis. Overall, this work shows that de novo purine synthesis is an important pathway in multiple myeloma and that it is reliant on glutamine to function properly.

Future directions for this work include performing stable isotope tracing experiments using labeled glutamine to determine if the PPAT KO's generated using CRISPR/Cas9 alter the

flux of glutamine through the de novo purine pathway. Reduction in PPAT expression could also change sensitivity of myeloma cell lines to myeloma therapies such as bortezomib, melphalan, and venetoclax.

Our current model for how glutamine is metabolized in myeloma is shown below. While glutamine is metabolized through several pathways, we believe that the most important role is fueling de novo purine synthesis.

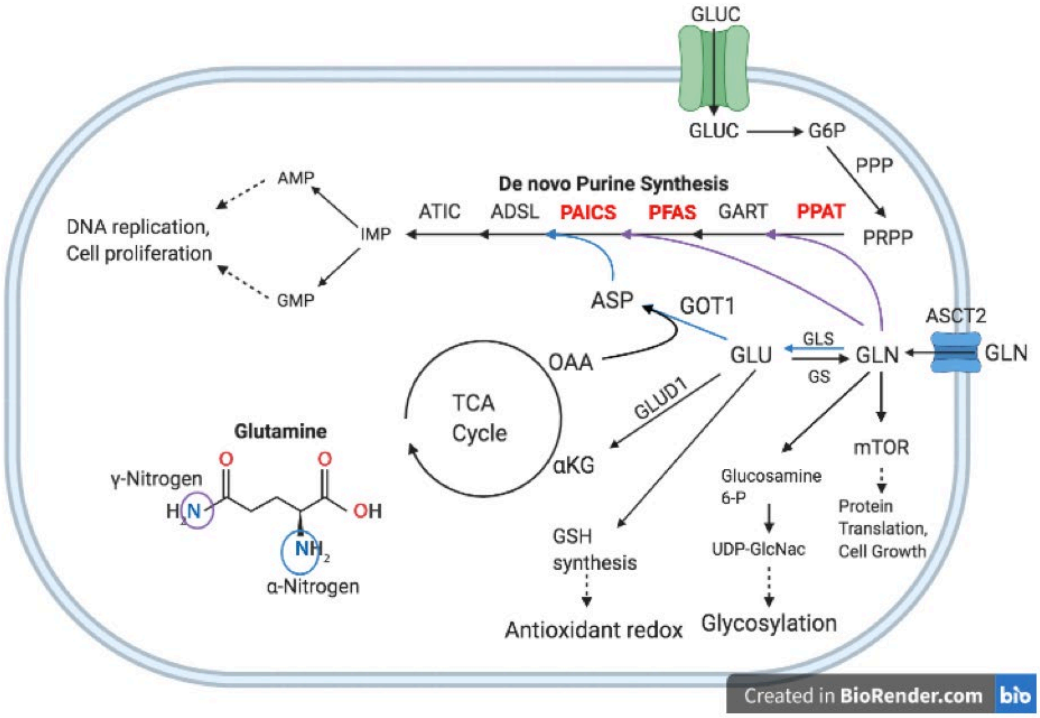

Figure 6: Overview of glutamine metabolism in multiple myeloma. Glutamine contributes to several different pathways including de novo purine synthesis, glutathione synthesis, mTOR signaling, TCA cycle, and glycosylation. Created using BioRender.

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