The Nutrients L-Glutamine and Glucose Have Unique Roles in B Lymphocyte Growth and Proliferation Responses

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THE NUTRIENTS L-GLUTAMINE AND GLUCOSE HAVE UNIQUE ROLES IN B LYMPHOCYTE GROWTH AND PROLIFERATION RESPONSES

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B cell activation is an energetically demanding process during which B lymphocytes undergo reprogramming and shift from a resting state to a highly proliferative, metabolically active state. Little is known about the metabolic reprogramming process or the role extracellular nutrients play in the activation response. Here we demonstrate that there are distinct requirements for the nutrients L-glutamine and glucose during activation. We show that cells activated in glucose-depleted conditions are still able to undergo growth and signaling events. In contrast, we show that extracellular Lglutamine is essential for all but the earliest activation events, and cells cultured in Lglutamine-deprived conditions are unable to enter the cell cycle. Consistently, we show that extracellular supplementation of the cell-permeable derivative of α-ketoglutarate (α-

KG), a glutaminolytic product, is able to rescue cell activation in the absence of glutamine. We also show the induction of the high affinity amino acid transporter ASCT2 is required for glutamine uptake following B cell receptor (BCR) crosslinking. Specifically, we found that halting glutamine uptake or processing by inhibiting ASCT2 or the glutaminolytic enzyme glutaminase causes activation defects that parallel those observed in glutamine deprived conditions, indicating a requirement for glutaminolysis during the very early stages of activation. We found that a-KG does not contribute to epigenetic remodeling, but is necessary for mammalian target of rapamycin complex 1

(mTORC1) activation. In turn, mTORC1 activity is required for upregulation of the glucose transporter Glut1 during the initial 24 hours of activation, as well as increased glucose uptake. These findings indicate a distinct metabolic profile that begins with glutamine uptake, and acts through mTORC1 signaling to later promote glucose uptake. Finally, we show that nutrients contribute to functional differentiation events during B cell activation. Glucose is required to support biogenesis of the endoplasmic reticulum as well as differentiation into plasma-like cells, while glutamine is required to support differentiation into IL-10 secreting regulatory B cell subsets. The requirement for glutamine for *in vitro* B10 cell differentiation is the first reported link between nutrient signaling and regulatory B cell development, and is a novel finding in the field.

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LIST OF ABBREVIATIONS

2-NBDG	2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-deoxyglucose
2HG	D-a-hydroxyglutaric acid
5mC	5-methylcytosine
ACLY	ATP-citrate lyase
ADCC	Antibody-dependent cell-mediated cytotoxicity
AKT	Protein kinase B
AMPK	AMP-activated protein kinase
APC	Antigen presenting cell
ASCT2	Alanine-cysteine-serine Transporter 2
ATP	Adenosine triphosphate
B10	IL-10 secreting B cell
BAFF	B-cell activating factor
BCR	B cell receptor
Be1	B effector type 1
Be2	B effector type 2
Blimp-1	B lymphocyte-induced maturation protein-1
BLNK	B-Cell Linker
Breg	Regulatory B cell
BSA	Bovine serum albumin
C-9	2-hydroxy-N-arylbenzenesulfonamide
CD family	Cluster of differnetiation family
CLP	Common lymphoid progenitor
DON	6-diazo-5oxo-L-norleucine
DTT	Dithiothreitol
E2F	E2 factor
EAA	Essential amino acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
FO	Follicular
FOXO	Forkhead box O
FSC	Forward Scatter
GC	Germinal center
GLS	Glutaminase

Glut1	Glucose transporter 1
GPNA	L-g-glutamyl-p-nitroanilide
GSH	Glutathione
GTP	Guanosine-5'-triphosphate
Н3	Histone 3
H3K9	Histone 3 lysine 9
HBSS	Hanks balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
HSC	Hematopoeitic stem cell
IDH1/IDH2	Isocitrate dehydrogenase 1/2
IFN-γ	Interferon gamma
Ig family	Immunoglobulin family
IL family	Interleukin family
IL4Ra	Interleukin 4 Receptor alpha
ITAM	Immunoreceptor tyrosine-based activation motif
JHDM	Jumonji domain-containing histone demethylase
LAMP-1	Lysosomal-associated membrane protein 1
LAT1	L-type amino acid transporter 1
LPS	Lipopolysaccharide
meth-AIB	Methylaminoisobutryic acid
MFI	Mean Fluorescence Intensity
MHC	Major histocompatibility complex
mTOR	Mammalian target of rapamycin
MZ	Marginal zone
NAD	Nicotinamide adenine dinucleotide
P-S6	Phospho-S6 protein
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PDH	Pyruvate dehydrogenase
PE	Phycoerythrin
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol myristate acetate
PMSF	Phenylmethylsulfonyl fluoride
PTK	Protein tyrosine kinase
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid Arthritis
RIPA	Radioimmunoprecipitation assay buffer

RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
TBS-T	Tris-buffered saline-tween
TCA	Tricarboxylic acid cycle
TdT	Terminal deoxynucleotidyl transferase
TET	Ten-eleven translocation enzyme
TGFβ	Transforming growth factor beta
Th1	T helper type 1
Th17	T helper type 17
Th2	T helper type 2
TLR	Toll like receptor
Treg	Regulatory T cell
TSC1/TSC2	Tuberous Sclerosis 1/2
α-KG	α-Ketoglutarate
β2Μ	β-2-microglobulin

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CHAPTER ONE

Introduction

The immune system is a tightly regulated system that serves to provide protection to an organism from outside pathogens while at the same time maintaining tolerance to self. To provide these functions, the immune system must be capable of quickly sensing and identifying foreign cells/tissues and substances, and acting in a coordinated manner to eliminate these invading pathogens. In mammals, the immune system consists of two branches, the innate and adaptive components, which cooperate to identify and eliminate pathogens. Innate immunity functions as the initial, nonspecific, arm of the immune system that is capable of quickly recognizing a diverse array of pathogens and then rapidly triggering an effector response. The adaptive branch of immunity provides a response that is highly specific, providing sterilizing immunity, and contributes to the development of immune memory.

The adaptive immune response is influenced by the innate immune system, and is essential when the early response provided by innate immunity fails to eliminate foreign pathogens. The adaptive immune response is mediated by lymphocytes, including CD8+ cytotoxic T lymphocytes (CD8+ T cells), CD4+ T helper lymphocytes (CD4+ Th cells), and B lymphocytes (B cells).¹ Cross-talk and intercellular communication between these cells, as well as other immune cell types, is essential for proper function of the adaptive immune response. B cells are tightly regulated throughout development and during maturity by signals from their environment, including antigen recognition at cell surface receptors, as well as by cytokine signals and ligation of co-stimulatory molecules by CD4+ Th cells. These signals regulate the survival, clonal expansion, and differentiation of mature B cells.

The mouse immune system in many ways closely resembles that of humans,

which provides a convenient platform from which to study cell types and processes that would otherwise be difficult or impossible to analyze. For this reason mouse models have been used for decades as an important tool in conjunction with the study of human immunology. Throughout this introduction, processes involved in immune cell development, signaling, and biochemical events shall be discussed that pertain to the immune systems of either mouse, human, or both.

B Lymphocyte Development

The development of white blood cells is a highly regulated process wherein hematopoietic stem cell (HSC) progenitors give rise to a diverse set of immune cell subsets.² During embryonic development, this process takes place in the fetal liver, and gives rise to the so called B1 subset of B cells that are enriched in the mucosal surfaces and peritoneal cavity.³⁻⁶ Throughout the course of adult life, HSC differentiation occurs in the bone marrow to produce additional B1 cells and the B2 subset of B cells.⁷ The B2 subset is enriched in the secondary lymphoid organs including spleen and lymph nodes, and will be the primary B cell population used in my investigations.^{8,9}

In the bone marrow, specific gene expression programs, cytokines, and cues from the environment and surrounding cells direct the differentiation of HSC progenitors into three major blood lineages: the erythroid, myeloid, and lymphoid lineages.¹⁰⁻¹² Development of B cells from HSCs occurs in distinct stages according to these signals, and is extensively regulated by expression of specific genes, the ability to produce functional antigen receptor, and the elimination of self-reactive B cells.¹³⁻¹⁵ To illustrate, prior to B cell lineage commitment, B cells exist in the bone marrow as a common lymphoid progenitor (CLP) subset that can give rise to B, T, and NK cells.¹⁶ Differentiation of CD34⁺ HSCs to CLPs is driven by the expression of *E2A*, *pu.1*, and *ikaros* genes, and characterized by expression of IL-7R-alpha and c-kit.^{17,18} Stromal cells in the bone marrow initiate the process of B cell commitment from CLPs by secreting IL-7, in part, to induce expression of terminal deoxynucleotidyl transferase (TdT) and V(D)J recombinase enzymes RAG1 and RAG2 that begin antigen-receptor gene rearrangement.^{17,19,20} The earliest committed B cells, progenitor B cells (pro-B), can be distinguished in the mouse by the surface expression of the CD45R isoform B220 and Class II MHC molecule along with the Pax5 and FOXO1 transcription factors.²¹ Finally, the cells undergo joining of the D-J segments on the immunoglobulin heavy (H) chain locus.²²

Pro-B cells transition to pre-B cells following the V segment joining on the H chain of the immunoglobulin heavy (H) chain locus (Figure 1). The pre-B cell also begins to express surrogate light chain (SLC) to produce a functional pre-BCR at the cell surface, which is critical to the cell's survival.²³ Successfully rearranged H chain together with Igα and Igβ heterodimer molecules expressed on the cell surface form a pre-BCR complex that functions to confirm activity and screen for self-reactivity and commit the pre-B cell to inactivate RAG enzymes and halt further H chain rearrangement.²⁴⁻²⁶ After several rounds of proliferation, cells initiate re-expression of the RAG enzymes for light (L) chain rearrangement and V-J joining.²⁷ B cells will then express surface IgM and become an immature B cell. This stage is highly susceptible to antigen binding, and if self-antigen is recognized in the bone marrow the immature B cell will undergo one of three fates: BCR induced cell death (negative selection),

inactivation/ anergy, or receptor editing to generate a non auto-reactive BCR.²⁸ If no self-antigen is bound, the immature B cell will exit the bone marrow as a transitional B cell.



Figure 1. B cell development, adapted and reprinted with permission from (29). B cell development is directed by a variety of growth factors and cytokines in the microenvironment, and regulated by the expression of functional BCR components.²⁹

Final stages of B cell development to the mature stage take place in the spleen. Although transitional B cells express surface IgM, they are unable to productively respond to antigen. Instead, antigen encounter can lead to anergy and apoptosis, allowing deletion of cells that respond to self-antigen in the periphery that may not have been present in the bone marrow.³⁰ If no autoreactivity occurs, the transitional B cell undergoes a differentiation into either a mature Marginal Zone (MZ) or Follicular (FO) B cell.^{8,31} The mature, resting B cell then resides in the spleen and awaits encounter with cognate antigen.

In the spleen and lymph nodes, mature B cells that encounter antigen can follow two distinct differentiation paths. Some B cells are capable of undergoing rapid, T cellindependent differentiation to become low-affinity plasmablasts. These cells mostly secrete IgM, and provide an earlier, but less specific antibody response. Activated B cell differentiation can also depend on interaction and costimulation by follicular dendritic cells (FDCs) and CD4+ Th cells.³² This signaling provided by CD4+ Th cells is essential for the process of class switching and affinity maturation of the BCR, and contributes to B cell survival, clonal expansion, and terminal differentiation.³³ BCR crosslinking alone is not enough to commit the cell to differentiation without additional signals from CD4+ Th cells. The interaction between B cells, CD4+ Th cells, and FDCs constitutes the germinal center (GC) reaction. B cells that have undergone affinity maturation and selection in the GC are able to provide an antibody response with maximum binding affinity for antigen. It is these high affinity B cells that are able to undergo terminal differentiation to long-lived antibody secreting plasma cells and circulating high affinity memory B cells.^{34,35}

Within the mouse, 5 x 10⁷ B cells are produced daily in the bone marrow, while only 2-5% survive to become mature circulating B cells.³⁶ This strict selection process is essential, as failure to remove or edit self-reactive B cells at any point can lead to a host of autoimmune disease states, including systemic lupus encephalitis (SLE) and rheumatoid arthritis (RA).^{37,38} In an adult mouse, there are approximately 10⁹ circulating mature B cells, with lifespans ranging from several days in the case of naïve cells up to a lifetime for terminally differentiated memory cells.³⁹⁻⁴¹

B cell activation and the role of B cell receptor signaling

Naïve mature B cells respond to environmental cues and signals and initiate G_1 entry during a process known as activation. This response is rapidly initiated following the interaction of antigen with surface receptors on a mature B cell. Following activation, B cells undergo metabolic reprogramming, allowing them to grow and

transition from the G_0 phase of the cell cycle to G_1 , characterized by an increase in biomass.^{42,43} Upregulation of metabolism and growth are also required for new DNA synthesis, which allows proliferation and clonal expansion.⁴⁴ The hallmarks of the metabolic shift following activation in B cells have been observed in a wide variety of proliferating cells, including activated T lymphocytes, cancer cells, and hepatocytes.⁴⁵

B cells are capable of recognition of specific antigen through a receptor-signaling complex located at the plasma membrane, the B cell receptor (BCR). The BCR is a membrane bound form of immunoglobulin (Ig) composed of two identical heavy chains and two identical light chains, covalently bound to form the complete receptor.⁴⁶ This extracellular portion of the receptor recognizes antigen at highly variable unique regions, providing specificity to the B cell clone. The BCR is also noncovalently associated with an Ig α and Ig β heterodimer, which provides the intracellular signal transduction response following antigen binding.47-50 Antigen recognition induces clustering of individual BCRs and signaling molecules into lipid raft microdomains, which act as a nucleation site for the initiation of signaling events.⁵¹⁻⁵³ Clustering of the receptors is enhanced through recognition of multivalent antigen by multiple BCR domains, and is essential for the propagation of signal intracellularly.⁵⁴ Following clustering into these microdomains a signaling cascade begins, initiated by tyrosine-based activation motifs (ITAMS) contained within the Ig α /Ig β dimer.^{50,55} ITAMs act as targets for Src family protein tyrosine kinases (PTKs) such as Lyn, and once phosphorylated can be bound by the SH2 domain of Syk kinase (Figure 2).⁵⁶⁻⁵⁹ Syk kinase can then recruit proteins such as B cell linker protein (BLNK), which activates major signaling pathways including



Figure 2. B cell activation by antigen recognition at the BCR, adapted and reprinted with permission from (75). Initiation of the activation response in B cells begins with antigen binding at the BCR. This signal is propagated into the cytoplasm by the activation of the ITAMs on the associated Ig α /Ig β heterodimer. Tyrosine kinases (SYK, BTK) and adaptor proteins (BLNK) are recruited to amplify the signal, which activates major signaling pathways such as PI3K/AKT signaling pathway, culminating in the activation of transcription factors (NFAT, MYC, JUN) that regulate cellular growth and proliferation.60

Ras/MAPK, PLC γ 2, and PI3K/Akt, eventually leading to metabolic re-programming, cytoskeletal re-organization, and the expression of transcription factors such as *c-myc*, *c-iun*, and NF κ B (Figure 2).⁶¹⁻⁶⁷

When the BCR is engaged, it triggers B cells through these signaling pathways to express a series of proteins that are necessary for a productive immune response. Because expression of these proteins is indicative of B cell activation and have established induction timelines, they are commonly used as activation markers. Some of the most frequently utilized early activation markers are CD86 and CD71.⁶⁸ CD86, also known as B7-2, acts as a costimulatory molecule for CD4+ Th cell activation by binding CD28 on the Th cell surface during formation of the immunological synapse.⁶⁹ The

protein is expressed at very low levels on resting B cells but is upregulated as early as 6 hours following BCR ligation, with maximum expression reached between 18 and 24 hours after activation.⁷⁰ CD71 is the transferrin receptor, necessary for importing iron in complex with transferrin by receptor-mediated endocytosis.⁷¹ It is expressed at high levels on all proliferating cells due to iron requirements, and is maximally induced in B cells 24-48 hours after activation.^{72,73}

Naïve B cells can be activated in vitro in several different manners, with different mechanisms of activation acting through different signaling mechanisms and eliciting different effector responses. The two main types of activation of B cells *in vivo* are Tdependent (TD), which includes limited crosslinking of the BCR by proteins and requires costimulation and cytokine signaling from CD4+ Th cells, and T-independent (TI), mediated by repetitive molecules that have either a strong propensity to crosslink the BCR (TI-2 type) or are recognized by germ line encoded receptors such as Toll-like receptors (TLR) (TI-1 type).⁷⁴⁻⁷⁶ TI activation, especially of the TI-2 type, can also be further augmented by CD4+ Th cell costimulation.^{77,78} These three traditional activation mechanisms can be replicated in vitro. In vitro, TD activation of B cells can be mimicked by adding anti-CD40 and IL4, to recapitulate the cytokine secretion and coligation that would normally occur during the interaction of CD4+ Th cells and B cells.⁷⁸⁻⁸⁰ To replicate TI-1 activation, LPS is the typical stimulant used in murine B cells, although it is not active in human B cells. LPS causes polyclonal activation through recognition by the TLR4 complex.^{81,82} These two methods of *in vitro* stimulation cause extensive proliferation, as well as differentiation, marked by endomembrane expansion and upregulation of plasma cells markers, culminating in Ig secretion.⁸³⁻⁸⁵ TI-2 activation can be imitated by addition of an anti-IgM Ab. This will result in a modest proliferation response with a high percentage of cells undergoing apoptosis, but with the benefit of synchronous cell cycle entry for ease of analysis.⁸⁶⁻⁸⁸ Apoptosis can be ameliorated by the addition of cytokines such as IL-4 or BAFF.^{77,89} It is also essential to only expose naïve B cells to the F(ab')2 fragment of antibodies for stimulation, as Fc receptors on the B cell surface will recognize whole Ab and initiate a negative regulatory response.^{90,91}

B cell effector functions

One of the most well described functions of B cells in the immune system is the production and secretion of antibody, however B cells in the periphery have diverse effector functions. Both high and low affinity secreted antibody is an essential component of immunity, and actually can trigger a varied set of effector functions.⁹² Antibodies are capable of directly binding antigen and coating the surface of an invading cell, in a process known as opsonization. This prevents the pathogen from invading and injuring other cells.^{93,94} Direct binding of pathogens also alerts other immune effector cells that are capable of recognizing the Fc' fragment of antibody to the presence of the invader, allowing them to recognize pathogen and mount a response, in a process known as antibody-dependent cell-mediated cytotoxicity (ADCC).⁹⁵⁻⁹⁷ Finally, antibodies can bind and fix (activate) complement to eliminate invading pathogens.^{98,99}

In addition to the functions of antibody itself, B cells perform cellular immune tasks as well. One of the other key functions of B cells in the periphery is to act as antigen presenting cells (APCs) to activate CD4+ Th cells.^{100,101} Unlike CD4+ Th cells,

B cells are capable of recognizing whole antigen in a native state at the BCR. Following antigen recognition of a protein-based antigen, B cells quickly begin endocytosis of BCR-antigen in complex, and shuttle antigen to the lysosome where it can undergo antigen processing and loading onto MHC-II to be redirected to the cell surface.¹⁰² MHC-II presents short peptide fragments that can be recognized by the TCR, prompting the CD4+ Th cell to co-ligate B cells at CD40 to form the immunological synapse, a close interface through which B and CD4+ Th cells can send signals such as through cytokine release (IL-4, -2, -5) and uptake.^{103,104} B cells are quite proficient at antigen processing and presentation, and act as professional APCs.

Similarly to CD4+ Th cells, B cells that have been activated can undergo differentiation into distinct types of effector cells to mediate specific responses. Plasma cells and memory cells are two of these effector types, and have been mentioned above. Additionally, newly activated B cells differentiate into three known classes of effectors that are characterized by their cytokine secretion profile.^{105,106} The first of these subtypes is the IL-10 and TGF β secreting B regulatory cell (Breg).^{106,107} B cells differentiate into the Breg subtype at low percentages following stimulation of BCR, CD40, or TLRs.¹⁰⁸⁻¹¹⁰ These cells are capable of CD4⁺ Th cell suppression, and have been shown to prevent induction of autoimmune disease in several mouse models.¹¹¹⁻¹¹⁶ Two B cell subtypes that positively modulate CD4⁺ Th cell activity have been identified with distinct cytokine secretion profiles.¹¹⁷ B effector 1 (Be1) cells are generated in response to antigen and Th1 cell signals, and secrete cytokines indicative of a type 1 immune response including interferon- γ (IFN- γ) and IL-12.¹¹⁷⁻¹²⁰ These cells can be induced without direct interaction with Th1 cells but in the presence of Th1 type

cytokines and TLR agonists.^{121,122} B effector 2 (Be2) cells are generated in response to antigen and Th2 stimulation, and secrete cytokines characteristic of allergy responses, including IL-4, IL-6, and IL-2.¹²³ Differentiation into the Be2 subtype requires IL-4 stimulation and CD40 and CD80/86 coengagement, and unlike Be1 cells is strictly dependent on T cell interactions (Th2 cells).¹²⁴

Although the study of B effector cells is a relatively new and under-represented field, encouraging results have already been observed in the use of effector B cell depletion as a therapy. B cell depletion has been shown to be an effective treatment of T cell mediated autoimmune diseases such as RA and SLE, independent of circulating auto-antibody levels, indicating a role for B effector cells in the regulation of the T cell mediated immune response^{111-114,116}. These findings highlight an important and previously unknown facet of B cell mediated immunity that deserves further investigation.

Transport and metabolism of nutrient molecules into B lymphocytes

Lymphocyte metabolism is now recognized as a critical regulator of normal lymphocyte responses during activation and differentiation (Figure 3).¹²⁵⁻¹²⁸ Resting lymphocytes are characterized by a metabolic profile that relies on a combination of glycolysis and fatty acid β oxidation, with the majority of ATP generated through the TCA cycle and oxidative phosphorylation. Both T and B cells increase glucose and glutamine uptake following activation, adopt a highly glycolytic and glutaminolytic profile, and limit fatty acid β oxidation to conserve lipids for membrane expansion during cellular growth.¹²⁸⁻¹³³ Interestingly, although both B and T cells increase glycolysis during activation, T cells

are more heavily glycolytic while activated B cells display a balanced increase in oxygen consumption and lactate production.¹³⁴ Together with increased mitochondrial mass this suggests a metabolic profile that still relies on TCA cycle activity and oxidative phosphorylation, however glycolysis is essential for certain functional activities including antibody production.^{131,134} Metabolic flux profiling has revealed that during the first several days following activation B cells utilize glucose-derived carbon and subsequently switch to preferentially utilize glutamine as a carbon source during the antibody production period.¹³⁵ It is possible that these two metabolites have different roles during the timeline of the B cell response, a theory that this thesis in part seeks to investigate.



Figure 3. The metabolic phenotypes of nonproliferating and proliferating cells differ. Nonproliferating cells utilize glucose, glutamine, and fatty acids equivalently to feed into the TCA cycle and generate ATP through oxidative phosphorylation. Proliferating cells primarily utilize glycolysis to generate ATP, and conserve fatty acids for membrane expansion while reserving glucose and glutamine as building blocks for the biosynthesis

of macromolecules need to support cellular growth and division.

In T cells, activation through the TCR and CD28 co-stimulation causes rapid upregulation of the high affinity glucose transporter, Glut1, in a PI3K and mTORC1 dependent manner.¹³⁶⁻¹³⁸ This transporter has also been observed to be upregulated in B cells following BCR crosslinking, and is PI3K dependent but only partially inhibited rapamycin treatment.¹³¹ Glucose uptake and glycolysis are also upregulated during B and T cell activation, and in B cells have been shown similarly to Glut1 expression to be PI3K dependent and partly mTORC1 dependent.^{131,137} Glycolytic activity is associated with increased inflammatory potential, and is highest in pro-inflammatory Th17 and Th1 subsets, and lower in regulatory T cells.¹³⁸⁻¹⁴¹ Increased glycolysis is additionally correlated to Glut1 expression in these cells, and transgenic Glut1 expression in T cells leads to hyperactivation with increased IL-2 and IFN-γ production.¹³⁸ Conversely, glucose depletion or inhibition of glycolysis has a suppressive effect on the inflammatory T cell response, resulting in decreased proliferation and IFN-y production. In B cells, inhibition of glycolysis is also functionally limiting, resulting in decreased antibody secretion both in vitro and in vivo.^{133,134} Glut1 is believed to be essential for glucose import in both T and B lymphocytes as Glut1 knockout studies have shown defects in activation similar to those observed during glucose depletion or inhibition of glycolysis.¹⁴²



Figure 4. Glucose can be metabolized to feed into *de novo* lipid biosynthesis pathways, adapted from (145) (permission not required for reprint). Glucose that has undergone glycolysis to generate pyruvate can enter the TCA cycle as acetyl-coA. Following conversion to citrate, and transport back into the cytoplasm, it can then be converted back to acetyl-coA by ATP citrate lyase and utilized for the synthesis of fatty acids, phospholipids, and cholesterol.¹⁴³

Once imported, glucose can be utilized as a carbon source for amino acid and phospholipid synthesis in addition to the generation of ATP and NADH (Figure 4).^{45,144-148} In B cells, glucose has been shown to contribute to *de novo* lipid synthesis in an ATP-citrate lyase (ACLY) dependent mechanism.¹³³ In this process, glucose is metabolized through glycolysis to produce 2 molecules of pyruvate. Pyruvate then enters the TCA cycle in the mitochondria and is converted to acetyl-CoA by pyruvate dehydrogenase complex (PDH), then further condensed with oxaloacetate to produce citrate by citrate synthase. Citrate then reenters the cytoplasm and is converted to acetyl-CoA (releasing oxaloacetate) by ACLY, and can act as a carbon source for the

biosynthesis of fatty acids and cholesterol through multistep metabolic reactions (Figure 4).¹⁴⁹⁻¹⁵²

Maintenance of glucose levels in proliferating cells is also critical to regulate AMPK activity. When ATP levels drop due to low intracellular glucose, AMPK becomes activated and negatively signals through mTORC1 to inhibit cellular growth until ATP levels can be restored.¹⁵³⁻¹⁵⁵ Interestingly, in T cells glutamine can act as an alternative fuel when glucose levels are limiting and provide metabolic intermediates and ATP generation through an alternative metabolic phenotype.¹²⁹

Transport and metabolism of L-glutamine in B & T lymphocytes

Lymphocytes in particular have been observed to rapidly increase glutamine import during the initial growth phase of activation, and are dependent on an extracellular supply of the amino acid to undergo an appropriate activation response.^{45,156,157} Glutamine uptake and metabolism have recently been characterized in T cells, however, virtually nothing is known about amino acid transport in B cells. This glutamine addiction has also been observed in several cancer cell types, and as such a recent interest in pathways of glutamine metabolism in proliferating cells has emerged.^{158,159}

Glutamine metabolism begins with the import into the cell through one of several high affinity transporters. Transporters of glutamine include SN1 and SN2 of system N, ATA1 and ATA2 of system A, and ASCT2 of system ASC.^{160,161} In T lymphocytes, ASCT2 has been demonstrated to be an important importer of glutamine; ASCT2 knockout mice display defects in differentiation of activated T cells into certain effector

subtypes, as well as decreased total numbers of B cells in the periphery, indicating that this transporter is likely important for B cell regulation as well.^{162,163}

Following uptake, glutamine can be immediately transported back out of the cell by transporters such as LAT1. This allows the import of other essential amino acids (EAAs) by antiport, which in turn activate mammalian Target Of Rapamycin Complex (mTORC), a key regulator of cellular growth.^{162,164,165} One of the initial steps in metabolizing glutamine is removal of the γ -nitrogen for incorporation into newly synthesized macromolecule precursors (Figure 5). Glutamine is an obligatory nitrogen donor for nucleotide synthesis of both purines and pyrimidines and is consequently indispensable for the production of new nucleotides needed for cell proliferation.^{149,158,166,167} This could suggest that cells deprived of glutamine are unable to proliferate due to the unavailability of nucleotides to synthesize new DNA during S phase. Studies have shown, however, that glutamine utilization rate in proliferating cells greatly exceeds nucleic acid synthesis, indicating that there are other pathways requiring nitrogen or carbon from glutamine.^{168,169} The γ -nitrogen group is also required for the hexosamine biosynthetic rate-limiting step in the pathway, catalyzed by amidotransferase.^{170,171} glutamine:fructose-6-phosphate This reaction forms glucosamine-6-phosphate, a precursor for N-linked and O-linked glycosylation reactions that are necessary for protein modification. Inhibition of these reactions can impair the ability of proteins to participate in cell signaling, and thereby inhibit cellular growth.¹⁷²

Removal of the γ -nitrogen can also produce ammonia and glutamate, when catalyzed by glutaminase (GLS). GLS is expressed at high levels in most proliferating

cells, including lymphocytes, and is induced by the transcription factor c-myc.^{157,173} High levels of GLS enable cells to rapidly generate glutamate from glutamine, and it is glutamate that acts as the substrate for reactions utilizing the carbon skeleton and α nitrogen of glutamine.¹⁶⁷

The carbon skeleton is mainly utilized via α -ketoglutarate generated from glutamate by either glutamate dehydrogenase or transamination. The α -nitrogen can be used in the synthesis of alanine or aspartate by transamination reactions catalyzed by alanine aminotransferase and aspartate aminotransferase.¹⁶⁹ Alanine is primarily used in protein synthesis, and only accounts for about 3% of the α -nitrogen use in lymphocytes. Aspartate transaminase is much more active, possibly because of the more varied uses of the amino acid in protein and nucleotide synthesis, as well as electron transfer reactions via the malate-aspartate shuttle.⁴²



Figure 5. Pathways of glutamine metabolism, adapted and reprinted with permission

from (176). Reactions utilizing the α -nitrogen are depicted in green; reactions utilizing the γ -nitrogen are depicted in orange, reactions utilizing glutamine derived carbon are depicted in pink, and energy generated from the metabolism of glutamine is depicted in blue.¹⁷⁴

Glutamate can also be used in the synthesis of GSH, a major cellular antioxidant that serves as a redox buffer against oxidative stress. Proliferating cells require adequate amounts of GSH to resist the oxidative stress generated by rapid metabolism.¹⁷⁵ Glutamate is one of three amino acids used in GSH synthesis, along with cysteine and glycine, and is thus necessary for synthesis of the antioxidant. High intracellular glutamate levels are also necessary for the import of cystine, the limiting substrate for GSH synthesis, by the X_c^- antiporter. This transporter exports glutamate in exchange for cystine, which is then converted to cysteine inside the cell and used in GSH production.¹⁵⁸

The α -ketoglutarate produced by the transamination reaction described above can be used for oxidation of glutamine's carbon skeleton in the mitochondria, via the TCA cycle. This is a key metabolic fate of glutamine in several proliferating cell types, including T lymphocytes and lymphoma cells, and can be a major source of cellular energy.^{146,157,158,176} Glutamine carbon can be oxidized to completion by exit from the TCA cycle as malate, followed by conversion to pyruvate and then acetyl-CoA, which can then reenter the cycle. Alternatively, pyruvate can be converted to lactate and secreted from the cell. This has been observed in tumor cell lines, and is referred to as "glutaminolysis", because of its parallels to the incomplete oxidation of glucose observed in the glycolytic phenotype of cancer cells.^{159,177} Conversion of glutamine carbon to pyruvate by malic enzyme is also a source of NADPH, a required electron donor for several metabolic reactions. In addition to ATP generation, this is another potential benefit oxidizing glutamine carbon through the TCA cycle.¹⁵⁸

As an alternative to complete cycling through the TCA cycle as a means to produce citrate from glutamine, reductive carboxylation of α -ketoglutarate to isocitrate can occur in the mitochondria, catalyzed by isocitrate dehydrogenase 1 (IDH1), or the cytosol, catalyzed by IDH2. This provides a shortcut for glutamine carbon to be converted to acetyl-coA and utilized in *de novo* lipid synthesis, which is necessary during cellular growth. In some glutamine addicted proliferating cells, glutamine has been discovered to actually contribute the majority of the carbons for lipid synthesis, while glucose is metabolized through glycolysis and eventually secreted from the cell as lactate.¹⁷⁸

An additional crucial role for L-glutamine derived α -ketoglutarate that has been established in proliferating tumor cells is to function as a substrate for the epigenetic modifying enzymes JHDM and TET. In these reactions, α -KG serves as an obligate methyl group acceptor during the demethylation reactions of 5-methyl-cytosine of DNA by TET enzymes, or during histone demethylation by JHDM.¹⁷⁹⁻¹⁸² In cancer cells, α -KG can be competitively inhibited by the onco-metabolite 2-hydroxy-glutarate (2HG), an analog that is produced by mutant IDH isoforms that binds the active site of these enzymes but cannot act as a methyl acceptor.¹⁸³⁻¹⁸⁵ This has been observed to affect epigenetic reprogramming, causing cancer cells to enter a de-differentiated state.

mTOR signaling and regulation of lymphocyte responses

Signaling through the mTOR pathway has been studied extensively in lymphocytes, and is known to be essential for both B and T lymphocyte responses.^{136,186-} 188 Emerging evidence suggests that an integral component of mTOR activity during lymphocyte responses is the coordination of cellular growth and metabolic reprogramming.¹⁸⁹⁻¹⁹¹ The mTOR serine/threonine kinase functions in mammals as a part of two distinct multi-protein complexes, mTORC1 and mTORC2, characterized by differences in activity and protein components (Figure 6).^{187,192} mTORC1 is comprised of the mTOR kinase along with the proteins mLST8, Deptor, PRAS40, and most distinctively Raptor, making it susceptible to inhibition by rapamycin.¹⁹²⁻¹⁹⁴ mTORC2 also contains mTOR kinase as the catalytic subunit, in addition to mLST8, DEPTOR, Sin1, Protor1/2, and the characteristic subunit Rictor, and is significantly less sensitive to rapamycin treatment, with inhibition only reported by prolonged or high doses in certain cell types.^{192,195,196} Both complexes are active as downstream effectors of the PI3K signaling pathway, and in B cells are turned on in response to mitogenic signals or BCR crosslinking.197



Figure 6. The proteins that make up mammalian target of rapamycin complex 1 (mTORC1) and 2 (mTORCS2), adapted from (189) (permission for reprint not required).¹⁸⁷

mTORC2 activity has been less well characterized than mTORC1 in lymphocytes, however it's functions in growth and downstream effectors appear to be significantly different. mTORC2 also acts downstream of PI3K, however detailed information on the mechanisms of activation and regulation is yet to be elucidated. Downstream targets include AKT, SGK1, and PKC- α , with AKT being the most extensively studied.^{189,198,199} Key roles of mTORC2 include regulation of cell metabolism through AKT, as well as cytoskeletal rearrangement.^{192,200} One of the most important functions of mTORC2 in B cells is the phosphorylation of the FOXO transcriptional repressor, promoting removal of FOXO and subsequent degradation in the cytoplasm, thus allowing cell cycle entry to proceed.²⁰¹ mTORC2 also has a role in effector differentiation of activated lymphocytes; in a RICTOR knockout mouse model, activated CD4⁺ T cells failed to undergo differentiation to the Th2 subset under polarizing conditions.¹⁹⁶ In B cells, mTORC2 deletion was found to upregulate c-Myc, resulting in E2F1 downregulation and increased apoptosis, revealing a new mechanism by which mTORC2 regulates cell survival.²⁰²


Figure 7. Pathways involved in mTORC1 regulation, adapted from (205) (permission for reprint not required). mTORC1 acts downstream of the PI3K/AKT pathway. Negative regulation by TSC1/TSC2 of Rheb inactivates mTORC1. When extracellular signals activate the PI3K/AKT pathway, TSC1/TSC2 releases Rheb, thus activating mTORC1 to phosphorylate downstream targets such as S6, and promote cell growth and proliferation.²⁰³

Quiescent T and B cells typically exhibit very low levels of mTORC1 activity, however it is rapidly induced following lymphocyte stimulation, with phosphorylation of downstream targets appearing as early as 30 minutes following receptor crosslinking.¹⁸⁶ Unlike mTORC2, regulatory mechanisms impacting mTORC1 activation are well understood, and although primarily elucidated in non-immune cell types, some have been demonstrated to operate in T cells. mTORC1 activity is inhibited by the tuberous sclerosis 1/2 (TSC1/TCS2) complex, a heterodimeric GTP-ase activating protein (GAP), downstream of PI3K/Akt (Figure 7).¹⁹² PI3K/Akt inactivates TSC1/TSC2, releasing Ras homolog enriched in brain (Rheb), a small GTP-ase that promotes mTORC1 activity. When active, the GAP activity of TSC1/TSC2 regulates the GTP bound state of Rheb, converting it to the inactive GDP bound state. TSC1/TSC2 are also regulated by AMPK; when ATP levels in the cell are low AMPK phosphorylates and activates the TSC complex to promote GAP activity and Rheb inhibition. The downstream targets of mTORC1 kinase activity are also well studied. Activated mTORC1 initiates phosphorylation of S6 kinase, 4EBP1, and several other downstream targets, culminating in transcriptional activation of genes involved in cellular growth, glycolysis, lipid synthesis, and inhibition of cellular autophagy. As these downstream targets are strictly dependent on upstream mTORC1 activity for phosphorylation, and are not involved in any cross-reactive pathways, they make excellent markers to experimentally assess the level of mTORC1 activity in the cell.¹⁹²

mTORC1 has been demonstrated in several cell types to be regulated by amino acid availability through a complex mechanism.¹⁶²⁻¹⁶⁵ In a mechanism initially discovered in U2OS cells, import of glutamine through the SLC1A5 (ASCT2) transporter was coupled to exchange for leucine through SLC7A5 (LAT1) (Figure 8).¹⁶⁴ This amino acid exchange was required for mTORC1 activity. Both ASCT2 and LAT1 knockout studies have independently shown that this exchange also plays a role in mTORC1 activity in T cells.^{162,165} Additionally, intracellular α -KG is essential for the activation of mTORC1 in certain cell types.^{204,205} In these studies, α -KG is required for the activity of small Rag-GTPases. These Rag-GTPases control recruitment of mTORC1 to the lysosomal surface where it can interact with and be activated by Rheb, although it is unclear if this mechanism is also active in lymphocytes.²⁰⁴ Unlike mTORC1, mTORC2 is not known to be regulated by nutrient availability, either amino acids or ATP levels.



Figure 8. Glutamine and leucine import through ASCT2 and LAT1 (SLC7a5) are coordinated to regulate mTORC1 activity, adapted and reprinted with permission from (206). In T cells this mechanism is essential for inflammatory T cell responses.²⁰⁶

CHAPTER TWO

Materials and Methods

Reagents and Antibodies

AffiniPure F(ab')₂ fragment goat anti-mouse IgM was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block) antibody (Ab), PE-conjugated rat anti-CD71 monoclonal antibody (mAb), APC-conjugated rat anti- CD86 mAb, APC-conjugated rat anti-mouse CD19, APC-conjugated IgG2a, κ isotype control, and PE-conjugated IgG2a, κ isotype control Abs were obtained from BD Pharmingen (San Jose, CA). Alexa Fluor® 647 conjugated rabbit Phospho-S6 Ribosomal Protein (Ser235/236) mAb and rabbit anti-histone H3 mAb, and rapamycin were obtained from Cell Signaling Technologies (Beverly, MA). Alexa Fluor® 488 conjugated rabbit anti-glucose transporter (Glut1) mAb, FITCconjugated mouse 5-methylcytosine Ab, and rabbit anti-histone H3 (tri-methyl K9) Ab were obtained from Abcam Inc (Cambridge, MA). Rabbit anti-ASCT2 Ab was obtained from Millipore (Billerica, MA). Alexa Fluor® 488 conjugated goat anti-rabbit IgG was obtained from Life Technologies (Carlsbad, CA). PE-conjugated rat anti-mouse IL-10 and monensin were from eBioscience (San Diego, CA). Goat anti-rabbit IgG-HRP Ab was obtained from Santa Cruz Biotechnology, Inc (Dallas, TX). 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-deoxyglucose (2-NBDG) was obtained from Life Technologies. Gamma-L-glutamyl-p-nitroanilide hydrate was obtained from MP Biomedicals (Santa Ana, CA). Lipopolysaccharide (LPS 0111:B4), 6-diazo-5oxo-Lnorleucine (DON), and di-methyl 2-oxoglutarate were obtained from Sigma Aldrich (Natick, MA). D- α -hydroxyglutaric acid (sodium salt) (2HG) was obtained from Cayman Chemical (Ann Arbor, MI). Compound-9 (C-9) was synthesized by AsisChem Inc. (Watertown, MA). All other reagents, unless noted otherwise, were obtained from Fisher Scientific (Pittsburgh, PA).

B Lymphocyte Isolation and Culture

Male BALB/cJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were house in the Boston College Animal Care Facility cared for and handled at all times in accordance with National Institutes of Health and Boston College Institutional Animal Care and Use Committee guidelines. Splenic B cells from mice at 8–12 weeks were isolated and purified by negative selection using a B cell isolation kit, MidiMACSTM separator, and LS column (Miltenvi Biotec Inc.). Small dense B cells were isolated via centrifugation through a discontinuous 72:65:50% Percoll gradient. Purified B cells were cultured in RPMI 1640 medium (Corning) plus 10% dialyzed fetal calf serum (dFCS) (Atlanta Biologicals), 2 mM L-glutamine, 10 mM HEPES, pH 7.4, 50 μ M β -mercaptoethanol, 50 units/ml penicillin, and 50 μ g/ml streptomycin. For glutamine free culture conditions, cells were cultured in L-glutamine free RPMI 1640 medium, plus 10% dFCS, 10mM HEPES, pH 7.4, 50 μ M β -mercaptoethanol, 50 units/ml penicillin, and 50 μ g/ml streptomycin. For glucose free culture conditions, cells were cultured in glucose-free RPMI 1640 medium plus 10% dFCS, 2 mM L-glutamine, 10 mM HEPES, pH 7.4, 50 μ M β -mercaptoethanol, 50 units/ml penicillin, and 50 μ g/ml streptomycin. B cells were activated in culture by the addition of 10 µg/mL F(ab')₂ fragments of goat anti-mouse IgM.

Cell Size Analysis

Following stimulation and culture as described in the figure legends, B cells (10⁶ cells) were collected, washed in ice-cold staining buffer (PBS containing 2% FCS), and evaluated using the Forward Scatter (FSC-A) parameter on a BD FACSCanto flow cytometer. Alternatively, to obtain quantitative measurements of average cell volume or cell diameter, cells were treated as described and analyzed using the Millipore Scepter 2.0 cell counter with 40 micron sensors.

Cell Lysate Preparation and Western Blotting

For AMPK and P-S6 analysis, following stimulation and culture under conditions specified in the figure legends, B cells (10^7 cells) were collected, washed in PBS, and solubilized in Triton X-100 lysis buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 0.1% Triton X-100) containing protease inhibitor cocktail, Phospho-Stop Phosphatase inhibitor cocktail, 1 µM PMSF, and 1 mM DTT for 20 minutes on ice with gentle mixing. Insoluble debris was removed by centrifugation at 14,000 xg for 20 minutes at 4 °C. For total Histone H3 and H3me3K9 analysis, following stimulation and culture under conditions specified in the figure legends, B cells (10^7 cells) were collected, washed in PBS, and solubilized in RIPA lysis buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and 140 mM NaCl) containing protease inhibitor cocktail, 1 µM PMSF, and 1 mM DTT for 20 minutes on ice with gentle mixing. Insoluble debris was removed by centrifugation at 14,000 xg for 20 minutes at 4 °C. Total cellular protein was quantified using the BioRad Protein Assay, and equal amounts of protein were loaded and separated on a 10%

(AMPK and S6) or 15% (H3 and H3me3K9) polyacrylamide SDS gel following the addition of 2x SDS sample buffer and 10 minutes at 100 °C. Protein was then transferred to an Immobilon-PVDF membrane, and blocked in TBS-T (20 mM Tris [pH 7.6], 137 mM NaCl, and 0.05% Tween 20) containing 5% Bovine Serum Albumin (AMPK and S6) or 5% dry nonfat milk (H3 and H3me3K9) for 60 min at room temperature. The membrane was then rinsed and incubated overnight at 4 °C with the described primary Ab at the manufacturer's recommended dilution. The membrane was washed 4x for 5 min in TBS-T and incubated with a 1:25000 dilution of anti-rabbit HRP conjugated secondary Ab, washed an addition 4x for 5 min and developed by ECL and autoradiography film.

Cell Viability Analysis

To analyze the percentage of viable cells, 10^6 B cells were collected, washed in ice cold PBS, and resuspended in PBS containing 50 µg/ml propidium iodide for 30 min (37 °C) and analyzed by flow cytometry using a FACSCanto cytometer. Cells that stained positive for propidium idodide were excluded from the viable population.

Cell Cycle Measurements and Protein Detection

B cells (10^6 cells) were collected, washed in ice-cold staining buffer (PBS containing 2% FCS), and resuspended in PBS containing 0.1% Triton X-100, 50 µg/ml propidium iodide, and 50 µg/ml RNase A for 30 min (37 °C). DNA content was measured by flow cytometry using a FACSCanto cytometer. FITC staining for total intracellular protein content was carried out with 10^6 B cells that were washed with ice-cold staining buffer

and then fixed and permeabilized with the Cytofix/Cytoperm kit according to the manufacturer's instructions; cells were stained with FITC and analyzed by flow cytometry. Cell size was analyzed using flow cytometry by measuring the mean forward scatter of 10^6 B cells that were collected, washed in ice-cold staining buffer, and resuspended in staining buffer. For S6-pS235/236 staining, 10⁶ B cells were collected, washed in ice-cold staining buffer and then incubated in 100 µl of staining buffer containing 2 µl of rat anti-mouse CD16/CD32 for 20 min. Cells were then fixed and permeabilized using the Cytofix/Cytoperm kit according to the manufacturer's instructions and stained with Alexa Fluor® 647 conjugated rabbit anti-S6-pS235/236 for 60 min (4 °C). Cells were washed three times in staining buffer and analyzed by flow cytometry. B lymphocyte-induced maturation protein-1 (Blimp-1) staining was carried out with 10⁶ splenic B cells that were washed with ice-cold staining buffer and then fixed and permeabilized with the transcription factor buffer set according to the manufacturer's instructions (BD Pharmingen); cells were stained with Alexa Fluor 647conjugated rat anti-mouse Blimp-1 (BD Biosciences) and analyzed by flow cytometry.

Surface Marker Staining and Flow Cytometry

For CD86 staining, 10^6 B cells were washed with ice-cold staining buffer (PBS containing 2% FCS,) and then incubated in 100 µl of staining buffer containing 2 µl of rat anti-mouse CD16/CD32 Ab for 20 min. Cells were then stained with APC-conjugated rat anti-mouse CD86 Ab or APC-conjugated rat IgG2a, κ isotype for 60 min (4 °C). Cells were washed three times with staining buffer and analyzed by flow cytometry. For CD71 staining, 10^6 B cells were washed with ice-cold staining buffer

and then incubated in 100 μ l of staining buffer containing 2 μ l of rat anti-mouse CD16/CD32 Ab for 20 min. Cells were then stained with PE-conjugated rat anti-mouse CD71 Ab or PE-conjugated rat IgG2a, κ isotype control for 60 min (4 °C). Cells were washed three times with staining buffer and analyzed by flow cytometry. For CD138 surface staining, 10⁶ splenic B cells were washed with ice-cold staining buffer (PBS containing 1% FCS, 0.1% NaN₃) and then incubated with 100 μ l of staining buffer containing 1.5 μ l of rat anti-mouse CD15/CD32 Ab (BD Biosciences) for 20 min. Cells were then stained with PE-conjugated rat anti-mouse CD138 Ab or PE-conjugated rat IgG2a, κ isotype control (BD Biosciences) for 45 min (4 °C). Cells were washed three times with staining buffer and analyzed by flow cytometry.

Glutamine Transport Assay

B lymphocytes were washed and resuspended at 4.0 x 10⁷ cells/ml in choline-Krebs-Ringer (choline-KRP) buffer (119 mM choline chloride, 5.9 mM KCl, 1.2 mM MgSO₄, 1.2 mM KHCO₃, 5.6 mM glucose, 0.5 mM CaCl₂, 25 mM choline bicarbonate, 0.15% (w/v) bovine serum albumin (BSA)) and incubated for 30 min at 37°C in order to deplete intracellular sodium and amino acid pools. Cells were then collected by centrifugation and resuspended in choline-KRP at 4.0 x 10⁷ cells/ml. Transport was initiated by mixing 200 ml of B cells with 200 ml of choline-KRR buffer plus 4 mCi/ml [³H] glutamine or 200 ml of sodium-KRP buffer (119 mM NaCl, 5.9 mM KCl, 1.2 mM MgSO₄, 1.2 mM KHCO₃, 5.6 mM glucose, 0.5 mM CaCl₂, 25, mM choline bicarbonate, 0.15% (w/v) BSA) plus 4 mCi/ml [³H] glutamine. After the appropriate time, 50 ml of transport buffer plus cells was loaded on to a prepared gradient containing a 200 ml bromododecane upper layer and a 40 ml 20% perchloric acid lower layer and centrifuged at 14,000 x g for 2 min in a fixed angle microcentrifuge. The two upper layers were removed and discarded, while the perchloric acid layer was placed into a liquid scintillation vial containing aqueous-sample-compatible liquid scintillation cocktail. Samples were quantitated using liquid scintillation spectrophotometry. To determine the contribution of system A to Na+-dependent [³H] glutamine uptake, glutamine transport was carried out in the absence and presence of the system A specific nonmetabolizable substrate methylaminoisobutryic acid (meth-AIB) at a concentration of 1 mM. In order to determine the contribution of the high affinity System ASCT2 to Na+-dependent glutamine transport, [³H] glutamine uptake was carried out in the absence and presence of 1 mM threonine or 1 mM leucine. ASCT2 exhibits specificity to a wide panel of zwitterionic amino acids such as threonine, serine and to a lesser degree bulky/branchchain amino acids such as leucine and valine.

Nutrient Transporter Detection

For ASCT2 expression, 10^6 B cells were collected, washed in ice-cold staining buffer and then incubated in 100 µl of staining buffer containing 2 µl of rat anti-mouse CD16/CD32 Ab for 20 min. Cells were then fixed in 4% paraformaldehyde and permeabilized in PBS containing 0.1% Saponin and stained with rabbit anti-ASCT2 Ab for 60 min (4 °C). Cells were washed three times in ice-cold staining buffer and incubated in Alexa Fluor® 488 conjugated goat anti-rabbit Ab for 45 min (4 °C). Cells were washed three times in staining buffer and analyzed by flow cytometry. For Glut1 expression, 10^6 B cells were collected, washed in ice-cold staining buffer and then incubated in 100 μ l of staining buffer containing 2 μ l of rat anti-mouse CD16/CD32 Ab for 20 min. Cells were then fixed in 4% paraformaldehyde and permeabilized in PBS containing 0.1% Saponin and stained with Alexa Fluor® 488-conjugated rabbit anti-Glut1 mAb for 60 min (4 °C). Cells were washed three times in staining buffer and analyzed by flow cytometry.

qPCR

cDNA was isolated using the μ MACS One-step cDNA kit and thermoMACS separator (Miltenyi Biotec). Integrated DNA Technologies PrimeTime® qPCR primers for mouse Slc1A5 (ASCT2) and β -2-micoglobulin (β 2m) consisting of the following sequences:

β2m forward, 5'-TTCAAATGAATCTTCAGAGCATCA-3';

β2m reverse, 5'-TCTATATCCTGGCTCACACTGA-3';

Slc1A5 forward, 5'-ACATGACTTGTCTTTGGGTTCA-3';

Slc1A5 reverse, 5'-GCTGGATTCCTTTCTAGATCTCG-3'.

The relative quantities of ASCT2 expression were calculated and normalized to β 2m expression using the $\Delta\Delta$ Ct values for each sample.

Nutrient Uptake Measurements

For glucose uptake was measurements, 10^6 activated B cells were washed in PBS and resuspended in glucose-free RPMI and incubated for 4 hours. 150 µg/mL of the fluorescent glucose analog 2-NBDG was added and cells were incubated for an additional 30 minutes. Cells were then washed in PBS and analyzed by flow cytometry.

Endoplasmic Reticulum Staining for FACS and Microscopy

For ER-TrackerTM staining by flow cytometry, 10^6 splenic B cells were washed with icecold PBS. Cells were then incubated with 1 µM ER-TrackerTM dye for 30 min (37 °C), washed three times with PBS, and analyzed by flow cytometry. In all experiments, analysis was carried out on the gated viable cell population. Flow cytometry was carried with a BD FACSCanto cytometer, and the data were analyzed by FACSDiva software (BD Biosciences). B cells (1 × 10⁶) were collected and washed with HBSS containing 2% FCS. Cells were then incubated with 5 mM ER-Tracker GreenTM dye (Invitrogen) in HBSS containing 2% FCS for 30 min. B cells were washed with HBSS containing 2% FCS and resuspended in RPMI 1640 medium, transferred to chambered coverglass and analyzed by confocal microscopy.

Antigen Receptor Endocytosis Staining

For analysis of antigen receptor endocytosis, 10⁶ splenic B cells were washed with ice-cold PBS. Unstimulated B cells were incubated with FITC conjugated goat anti-mouse IgM on ice for 10 minutes. Cells were washed in ice cold staining buffer to remove excess antibody, and incubated at 37 °C for indicated time periods. Cells were then fixed and permeabilized with the BD Cytofix/Cytoperm kit according to the manufacturer's instructions, and stained with APC conjugated rabbit anti-mouse LAMP-1 for 60 minutes on ice. Cells were then washed 3 times in ice-cold staining buffer and analyzed by confocal microscopy for colocalization of antigen receptor and LAMP-1. Cells in media were mounted in ProLong Gold (Life Technologies, P36930) and imaged

with an APOCHROMAT 40X, 1.4 NA objective with a 3.0-X optical zoom for all images on a Zeiss 700 LSCM.

Analysis of IL-10 Production

Intracellular analysis of IL-10 production was carried out according to published methods.²⁰⁷ Purified B cells were cultured at a density of 2 x 10⁶ cells/ml in complete media, media lacking glutamine, or complete media containing 5 mM GPNA and stimulated with 10 µg/ml LPS for 48 hours. For the final 5 hours of stimulation, cells were treated with 50 ng/ml PMA, 500 ng/ml Ionomycin, and 2 μ M monensisn. 10⁶ B cells were collected, washed in ice-cold staining buffer and then incubated in 100 μ l of staining buffer containing 2 µl of rat anti-mouse CD16/CD32 for 20 min, followed by staining with APC-conjugated rat anti-mouse CD19. Cells were then fixed and permeabilized using the Cytofix/Cytoperm kit according to the manufacturer's instructions and stained with PE conjugated rat anti-mouse IL-10 for 60 min (4 °C). Cells were washed three times in staining buffer and analyzed by flow cytometry. To quantitate secreted IL-10, isolated B cells were cultured at a density of 4×10^5 cells in 0.2 ml of indicated media in a 96 well flat-bottom plate and stimulated with 10 µg/ml LPS for 72 hours. Cell culture supernatant was analyzed for IL-10 concentration using the BD Pharmingen IL-10 OptEIA ELISA kit according to the manufacturer's protocol.

CHAPTER THREE

Differential requirements for glutamine and glucose in B cell activation

RESULTS

Extracellular L-glutamine and glucose are dispensable for induction of very early activation surface markers

I initially sought to characterize nutrient requirements during very early stages of B cell activation. Early evidence indicates the importance of extracellular L-glutamine and glucose during lymphocyte activation, however it has not been established whether these nutrients are interchangeable, or what the specific role of each nutrient is during the B cell response.^{42,73,149} To determine the nutrient requirements we compared the expression of activation induced surface markers and growth related changes in B cells cultured in nutrient replete (complete) media to B cells cultured in the absence of Lglutamine or glucose. We chose to focus our analysis on expression changes in molecules that are known to have a functional role during B cell activation. Expression of CD86, the T cell costimulatory molecule B7-2, was upregulated in B cells stimulated with 10 µg/mL (Fab')₂ anti-mouse IgM (anti-Ig) for 24 hours as compared to the expression in resting cells (Figure 1). This upregulation was unaffected by the availability of extracellular L-glutamine or glucose. CD86 is a very early surface marker for activation of B cells, with maximal upregulation induced 12-24 hours following B cell receptor (BCR) crosslinking.⁶⁹

Extracellular L-glutamine but not glucose is required for induction of later activation surface marker CD71 and growth related activation events

The transferrin receptor, CD71, is an early activation marker with maximal upregulation occurring at approximately 24 hours after BCR crosslinking. CD71 is

expressed on all proliferating cells and is essential for the import of iron via transferrin.^{71,73} Surface CD71 was also increased following 24 hour stimulation with anti-Ig as compared to resting, non-stimulated B cells in both nutrient replete conditions and glucose depleted conditions, although to a lesser extent. Cells stimulated in Lglutamine depleted conditions did not exhibit significant CD71 upregulation as compared to resting cells (Figure 2). In addition to changes in expression of cell surface markers, activated B cells also undergo growth related changes, including increased cell size and upregulation of protein synthesis, later followed by S phase entry and DNA synthesis. Following stimulation with anti-Ig, both B cell populations cultured in complete media and in the absence of glucose were able to increase cell size, as determined by both forward scatter and average cell diameter, whereas cells stimulated in the absence of L-glutamine did not increase cell size as compared to naïve cells (Figure 3a, 3b). One of the growth events associated with B cell activation is an increase in protein synthesis. Resting B cells are transcriptionally and translationally inert, however both mRNA and protein synthesis are elevated following BCR crosslinking.²⁰⁸ As both glucose and glutamine can be metabolized to produce amino acids such as glycine, serine, cysteine, alanine, asparagine, and proline, which may be essential for the synthesis of new proteins, I sought to examine whether intracellular protein levels would be affected by glucose or glutamine depletion. A simple and easy method for roughly quantifying intracellular protein is to permeabilize cells and stain with fluorescein isothiocyanate (FITC). FITC is widely used to covalently label proteins via reaction with the terminal amino groups and primary amines of proteins.^{209,210} Total intracellular protein content was measured by intracellular FITC labeling, and was shown to increase following stimulation in cells cultured in complete media. The increase in total protein content was reduced in L-glutamine deprived cells, and to a lesser extent in glucose deprived cells, however was still elevated as compared to naïve cells (Figure 4).

Extracellular L-glutamine does not contribute to cell viability

B cells stimulated with anti-Ig alone rapidly undergo apoptosis and typically lose 50% of viable cells after just 24 hours, with even lower viability during extended times in culture.⁷⁴ To examine whether nutrient availability had an impact on cell survival following BCR crosslinking, cells were analyzed by propidium iodide (PI) exclusion assay after 24 hours of culture in complete media, glutamine depleted media, or glucose depleted media and compared to naïve cells cultured for the same time period. PI is a DNA intercalating agent that only fluoresces when in contact with DNA. As it is a membrane-impermeable dye, it will only be able to enter cells and nuclei that have lost membrane integrity as associated with cell death, making it an especially useful tool to discriminate live from dead cells by flow cytometry. Interestingly, although lack of extracellular glutamine led to no significant change in cell viability after 24 hours, lack of extracellular glucose led to a decrease in viability compared to cells in complete media (Figure 5a). To analyze time-dependent changes in viability, a time course assay was performed to compare viability of glutamine deprived, complete media, and unstimulated / naïve cells after 4, 8, 12, and 24 hours in culture. Again, B cells stimulated in glutamine depleted media exhibited only a marginal decrease in cell viability during the 24 hour period following BCR crosslinking (Figure 5b).

Extracellular L-glutamine is required for cell cycle entry; cell cycle entry is only slightly impaired in the absence of extracellular glucose

Activated B cells in culture begin entry into S phase approximately 48 hours after stimulation, and previous studies have shown an essential requirement for L-glutamine in order for lymphocytes to undergo DNA synthesis.^{149,166,211} To determine whether glucose is also essential for this process, we examined intracellular DNA content of B cells 48 hours after anti-Ig stimulation by PI staining and flow cytometric analysis. We found that glucose deprived cells were still able to increase DNA content, although to a lesser extant than cells cultured in complete media. L-glutamine deprived cells were unable to increase DNA content as compared to naïve cells (Figure 6). Together with the growth related differences in glucose and glutamine deprived B cells, these data indicate a clear difference between the effects of extracellular L-glutamine and glucose on early B cell activation events. The severe impairment of growth related changes in L-glutamine deprived cells indicates a block at G₁ entry, whereas glucose appears to be dispensable for cell cycle entry and progression through S phase.

Increased IL4Ra expression is dependent on both extracellular L-glutamine and glucose

Having determined distinct requirements for glucose and glutamine during B cell activation, I proposed to establish a functional marker that could be used to FACS sort and purify cells that had gone through the early activation growth stages that require glutamine. These cells could then be placed back in culture in glutamine depleted media to determine whether glutamine was required for early stages only. In order to sort cells and have them remain viable the marker must be expressed on the cell surface so as not to require fixation for staining, and must clearly distinguish between positive and negative populations, which ruled out CD71 as a good candidate marker. Following BCR crosslinking, B cells rely on expression of surface receptors for IL4, IL4Ra within the first 24 hours.²¹² IL4Ra recognizes IL-4 and IL-13 and regulates, for example, IgE production in B cells.²¹³⁻²¹⁵ IL-4 is a pro-survival cytokine released by T cells in response to B cell antigen presentation that inhibits BCR crosslinking induced apoptosis through signaling through STAT6.^{132,216} Interestingly, IL4R α expression has also been found to be essential for the differentiation of activated B cells into the B-effector 2 (Be-2) subtype, characterized by secretion of IL-2, IL-4, and IL-6.¹⁰⁶ Differentiation into the Be-2 effector type is mediated by obligatory signaling from T cells, including IL4 secretion. To determine whether IL4R α expression was glucose or glutamine dependent, naïve splenic B cells were stimulated with 10 µg/mL anti-Ig for 24 hours in complete media, media lacking glucose, or media lacking glutamine, and compared to naïve cells for IL4R α expression as analyzed by flow cytometry. IL4R α expression was maintained but not increased in cells following activation, however this expression was decreased by either glucose or glutamine depletion (Figure 7). Interestingly, although this receptor was analyzed during the same timeline as our other early activation markers, its expression was equally glucose and glutamine dependent. This indicates a mechanism of induction that is distinct from the growth related events characterized as differently affected by glucose or glutamine depletion.

Low concentrations of extracellular L-glutamine are sufficient to fully restore early activation events

Having determined that there are distinct differences between the requirements for extracellular glucose and glutamine during the B cell activation, I next sought to determine whether these effects were dependent on the concentration of extracellular nutrients. Cell culture media typically contains high concentrations of nutrients well beyond concentrations that would be physiologically relevant or typically encountered in vivo^{129,159} However, early studies in lymphocytes indicated that these high concentrations of nutrients were necessary to produce a vigorous proliferation response *in vitro* and therefore were adopted as standard culture media for lymphocyte activation in culture. Standard B cell culture media (RPMI1640) contains 11 mM glucose and 2 mM glutamine, and typically contains additional supplemented 2 mM glutamine for a final concentration of 4 mM glutamine. To determine the effect of lower concentrations of these two nutrients on activation markers and cell cycle entry, splenic B cells were stimulated in media containing either 0 mM, 0.5 mM, or 4 mM glutamine, or 0 mM, 1 mM, or 11 mM glucose. The 4 mM glutamine media and 11 mM glucose media were included to control for potential effects of adding the nutrients back to RPMI media that did not initially contain any amount of either glucose or glutamine. Expression of CD86 was analyzed by flow cytometry surface staining, and was found to not be detectably affected by concentration of either glucose or glutamine (Figure 8). This correlates with the original finding that nutrient depletion did not negatively impact CD86 induction following BCR crosslinking. Although error bars appear large for this experiment, this is due to the dim staining, and representative histograms accurately depict that there are no visible differences in expression across the sample conditions. Surface CD71 expression was also analyzed by flow cytometry, as its induction had been previously found to be glutamine and glucose dependent, with differences in inhibition between the Interestingly, CD71 expression was fully restored by low concentrations of two. glutamine, however low concentrations of glucose were not sufficient to restore maximal upregulation (Figure 9). When cell size was analyzed by flow cytometry using the FSC parameter, low concentrations of glutamine were again sufficient to fully restore cell size increase following anti-Ig stimulation. Inhibition of cell size increase under glucose limiting conditions was minimal even under complete glucose depletion, but appeared to be concentration dependent, with cell size increasing more with each increasing glucose concentration (Figure 10). This in combination with the CD71 data suggests that glutamine requirement may act in a "threshold" manner, with low concentrations being sufficient to fully restore activation events, while glucose associated defects are directly regulated by the concentration of glucose. To analyze cell cycle entry, splenic B cells were activated with anti-Ig and the cytokine IL-4 for 48 hours and DNA content was analyzed by flow cytometry. IL-4 was included in the activation media to increase viability at the 48 hour time point, as many cells undergo apoptosis by this time point when stimulated with anti-Ig alone. Additionally, IL-4 costimulation increases the total percentage of cells that enter S phase, allowing me to better observe any changes in percentage associated with nutrient concentration that may have been more difficult with very low maximum percentage cell cycle entry. Similarly to the earlier growth related activation events (CD71 and cell size), cell cycle entry was fully restored by low concentrations of extracellular glutamine (Figure 11). In fact, previous studies in total lymphocyte populations indicated that concentrations as low as 0.1 mM glutamine restored proliferation and high concentrations of glutamine did not lead to increased proliferation.^{149,166} The results shown here confirm that this finding remains in effect in purified B cell populations. Conversely, cell cycle entry as measured by percentage of cells in S phase was dependent on the concentration of glucose, with 0 mM glucose resulting in the lowest percentage of S phase cells, and 11 mM glucose resulting in the highest. These findings reveal previously unknown distinctions between the requirements for glucose and glutamine during B cell activation. Altogether, these data indicate that the glutamine requirement occurs earlier than the glucose requirement, and is indicative of a G₁ block, and is a threshold effect with low concentrations being sufficient. Under glucose limiting conditions, although early effects are observed they are not as severe as observed under glutamine depletion, and is required in a directly concentration dependent manner.

FIGURES AND LEGENDS

Figure 1. Extracellular L-glutamine and glucose are not required for CD86

upregulation during B cell activation. Naïve B cells were activated with 10 μg/mL anti-Ig and cultured in either complete (nutrient replete) media, media lacking glucose, or media lacking L-glutamine for 24 hours and compared to naïve cells. Cells were collected and then analyzed by flow cytometry for surface expression of CD86. Data represents analysis of live gated B cells and is expressed as the percentage of cells that stained positive for CD86 expression. The data are representative of 2 independent experiments. Error bars represent standard deviation from the mean. Shown below are representative histograms of CD86 expression for cells cultured in complete media (red), media lacking glucose (green), or media lacking L-glutamine (blue) overlaid with expression on naïve cells (grey).





Figure 2. Expression of the later activation marker CD71 in response to nutrient depletion. Naïve B cells were activated with 10 μg/mL anti-Ig and cultured in either complete (nutrient replete) media, media lacking glucose, or media lacking L-glutamine for 48 hours and compared to naïve cells. Cells were collected and then analyzed by flow cytometry for surface expression of CD71. Data represents analysis of live gated B cells and is expressed as Mean Fluorescence Intensity (MFI). The data are representative of 3 independent experiments. Error bars represent standard deviation from the mean. Shown below are representative histograms of CD71 expression for cells cultured in complete media (red), media lacking glucose (green), or media lacking L-glutamine (blue) overlaid with expression on naïve cells (grey).

Figure 2.



Figure 3. BCR-mediated cell size increase is inhibited by L-glutamine deprivation. Naïve B cells were activated with 10 μ g/mL anti-Ig and cultured in either complete (nutrient replete) media, media lacking glucose, or media lacking glutamine for 24 or 48 hours and compared to naïve cells. *A*. Cells were analyzed by flow cytometry for cell size by mean Forward Scatter (FSC) as described in Materials and Methods, gated on live cells. Data is expressed as Mean FSC. Results are representative of three independent experiments. Error bars represent standard deviation from the mean. *B*. Cells were analyzed after 24 or 48 hours for mean cell diameter by the Millipore ScepterTM. The data are represented as mean cell size in μ m (Naïve shown in light blue, complete media shown in red, 0 mM glucose shown in dark blue, 0 mM glutamine shown in green).

Figure 3.





B

Cell Diameter



Figure 4. Intracellular protein content is dependent on extracellular L-glutamine. B cells were activated with 10 μg/mL anti-Ig and cultured in either complete (nutrient replete) media, media lacking glucose, or media lacking L-glutamine for 24 hours and compared to naïve cells. Cells were collected and stained with FITC as described in the Materials and Methods. Data is expressed as Mean Fluorescence Intensity (MFI). Results are representative of 3 independent experiments. Error bars represent standard deviation from the mean. Shown below are representative histograms of FITC total protein staining for cells cultured in complete media (red), media lacking glucose (green), or media lacking L-glutamine (blue) overlaid with expression on naïve cells (grey).

Figure 4.



Figure 5. Cell viability is not measurably affected by L-glutamine deprivation. Naïve B cells were activated with 10 μ g/mL anti-Ig and cultured in either complete (nutrient replete) media, media lacking glucose, or media lacking L-glutamine and compared to unstimulated naïve cells. Cells were analyzed by flow cytometry for viability by PI exclusion. *A*. Cells were cultured for 24 hours and analyzed for viability. Data are expressed as percentage of viable cells from analysis of 10,000 cells. Results are representative of two independent experiments. *B*. Cells were analyzed at 4, 8, 12, and 24 hours following stimulation (Complete media shown in blue, 0 mM glutamine shown in red, unstimulated cells shown in green). Data are expressed as percentage of viable cells from analysis of 10,000 cells. Results are representative of two independent experiments. Error bars represent standard deviation from the mean.

Figure 5.





B



Figure 6. Cell cycle progression in the absence of glucose. Naïve B cells were activated with 10 μ g/mL anti-Ig and cultured in either complete (nutrient replete) media, media lacking glucose, or media lacking L-glutamine for 48 hours and compared to naïve cells. Cells were collected and stained for DNA content by PI after fixation, permeabilization and RNAse treatment. Cells were analyzed by flow cytometry; results are representative of 3 independent experiments. Data is represented as the percentage of cells in G₀/G₁ (shown in blue), S (shown in red), and G₂/M (shown in green) stages of the cell cycle.

Figure 6.



Figure 7. IL4Ra expression is downregulated in the absence of extracellular glucose

or L-glutamine. B cells were activated with 10 µg/mL anti-Ig and cultured in either complete (nutrient replete) media, media lacking glucose, or media lacking L-glutamine for 24 hours and compared to naïve cells. Cells were analyzed by flow cytometry for surface expression of IL4Ra, gated on live cells. Data is expressed as Mean Fluorescence Intensity (MFI). Results are representative of two independent experiments. Error bars represent standard deviation from the mean.
Figure 7.



Figure 8. BCR-induced CD86 upregulation is not significantly affected by nutrient concentration. Naïve B cells were activated with 10 µg/mL anti-Ig and cultured in media containing 0 mM, 1 mM, or 11 mM glucose, or media containing 0 mM, 0.5 mM, or 4 mM L-glutamine for 24 hours and compared to naïve cells. Cells were analyzed by flow cytometry for surface expression of CD86, gated on live cells. Data is expressed as the percentage of cells that stained positive for CD86 expression, results are representative of two independent experiments. Error bars represent standard deviation from the mean. Shown below are representative histograms of CD86 expression at different nutrient concentrations (blue) overlaid with expression on naïve cells (grey).

Figure 8.



Figure 9. Relatively lower concentrations of extracellular L-glutamine are sufficient to restore CD71 upregulation. Naïve B cells were activated with 10 μg/mL anti-Ig and cultured in media containing 0 mM, 1 mM, or 11 mM glucose, or media containing 0 mM, 0.5 mM, or 4 mM L-glutamine for 48 hours and compared to naïve cells. Cells were analyzed by flow cytometry for surface expression of CD71, gated on live cells. Data is expressed as Mean Fluorescence Intensity, results are representative of two independent experiments. Error bars represent standard deviation from the mean. Shown below are representative histograms of CD71 expression at different nutrient concentrations (blue) overlaid with expression on naïve cells (grey).

Figure 9.



Figure 10. Low concentrations of extracellular L-glutamine are sufficient to restore cell size. Naïve B cells were activated with 10 µg/mL anti-Ig and cultured media containing 0 mM, 1 mM, or 11 mM glucose, or media containing 0 mM, 0.5 mM, or 4 mM L-glutamine for 24 hours and compared to naïve cells. Cells were analyzed by flow cytometry for cell size measure by mean forward scatter, gated on live cells. Data is expressed as Mean Forward Scatter, results are representative of two independent experiments. Error bars represent standard deviation from the mean. Shown below are representative histograms of forward scatter at different nutrient concentrations (blue) overlaid with expression on naïve cells (grey).





Figure 11. Low concentrations of extracellular L-glutamine are sufficient to restore cell cycle entry. B cells were activated with 10 μ g/mL anti-Ig and IL4 and cultured in media containing 0 mM, 1 mM, or 11 mM glucose, or media containing 0 mM, 0.5 mM, or 4 mM L-glutamine for 48 hours and compared to naïve cells. Cells were cultured for 48 hours and stained for DNA content by Propidium Iodide after fixation, permeabilization and RNAse treatment. Cells were analyzed by flow cytometry; results are representative of two independent experiments, error bars represent standard deviation from the mean. Data is represented as the percentage of cells in G₀/G₁ (shown in blue), S (shown in red), and G₂/M (shown in green) stages of the cell cycle.

Figure 11.



CHAPTER FOUR

ASCT2 mediated L-glutamine uptake and glutaminolysis are essential for

mTORC1 activity

RESULTS

Glutamine transport in B cells occurs in a sodium-dependent manner via System ASC

Having determined that extracellular L-glutamine is required at a very early stage of B cell activation, I next investigated the mechanism by which uptake into the cell is achieved. There are several transporters capable of translocating L-glutamine as a substrate, most notable being transporter systems A, L, and ASC. Systems A and ASC are sodium-dependent carriers, while system L is sodium-independent.^{161,217} To narrow down which transport system was primarily responsible for L-glutamine import, Lglutamine uptake was evaluated in the presence or absence of external sodium. Splenic B cells were cultured with phorbol myristate acetate (PMA) plus ionomycin overnight to induce activation.²¹⁸ For these studies, B cells were stimulated with PMA and ionomycin in order to increase the total number of cells stimulated *in vitro*.²¹⁸ Uptake of ³H] glutamine was measured at 45, 90, 120, and 180 seconds in choline KR buffer with or without sodium. Uptake of L-glutamine occurred in both sodium containing buffer and sodium free buffer, however was greater in sodium containing buffer, indicating that uptake primarily occurs in a sodium dependent manner (Figure 12). In order to further determine the transport system mainly responsible for L-glutamine uptake, inhibitors specifically on either system A, L, or ASC that act were utilized. Methylaminoisobutyric acid (MethAIB) is a non-metabolizable transport-specific analog inhibitor of system A.²¹⁹ System ASC is a strict stereoselective transporter with site specificity best fitted to threonine. System L exhibits broad specificity and preferential transport of leucine, however it is sodium-independent, and thus is likely not the primary mediator of glutamine uptake.²¹⁷ Glutamine uptake increased 5-fold in cells stimulated with PMA and ionomycin when compared to untreated cells, in agreement with data from early studies demonstrating that lymphocyte stimulation induces an increase in glutamine consumption.^{42,149,166} Uptake of glutamine remained high in the presence of 1 mM MethAIB, demonstrating that system A is not a significant glutamine importer. Glutamine uptake decreased 11-fold in the presence of 1 mM threonine, and in the presence of 1 mM leucine glutamine uptake decreased 4-fold and was similar to the level seen in cells cultured in medium alone (Figure 13). Although competitive inhibition of system L decreased glutamine uptake 4x, this is more likely do to the obligate antiport activity of this system.^{164,220} In exchange for leucine and other amino acids, system L transporters will selectively export glutamine from the cell, so a high concentration of extracellular leucine would most likely drive heightened export of glutamine. These results, in context with uptake occurring in a primarily sodium dependent manner, indicated that system ASC is the primary mediator of glutamine uptake in activated B cells.

ASCT2 is upregulated following BCR engagement in a time dependent manner at both the mRNA and protein levels

Within system ASC family, the transporter ASCT2 (Slc1A5) has a high affinity for glutamine, and has been shown to be a critical importer of glutamine in activated T lymphocytes and lymphoma cell lines.^{160,217,221-223} To confirm ASCT2 involvement in activation associated glutamine uptake, I evaluated whether ASCT2 is upregulated following BCR crosslinking. ASCT2 expression was measured at the mRNA level by qPCR in samples at 0, 6, 12, 18, and 24 hours in culture with anti-Ig, and expression

levels were normalized to β -2 microglobulin (β -2M) using $\Delta\Delta$ Ct values. Due to the inherently low transcription level in resting B cells, traditional methods of mRNA isolation/cDNA generation failed to yield reproducible results when ASCT2 expression levels were analyzed by qPCR. Therefore, I optimized an "on column" method (Miltenyi Biotec) in which whole cell lysate is applied over a column to isolated mRNA, and the column is then heated to cycle through the RT-PCR reaction without the need for tube transfer (detailed protocol available through miltenyibiotec.com). This system serves to minimize sample loss and ensure reproducible quantities of cDNA are generated from even low initial concentrations of mRNA. While quiescent B cells express low levels of ASCT2, expression was found to increase in a time-dependent manner following B cell stimulation, with increased expression as early as 6 hours post BCR crosslinking and peak expression occurring after 18-24 hours (Figure 14). To confirm expression at the protein level, I developed a flow cytometry-based protocol. ASCT2 is a multispanning membrane protein that is highly susceptible to degradation following cell lysis. This feature of the protein makes it very difficult to perform quantitative analysis of protein by SDS-PAGE and western blot. There are no commercially available antibodies available that recognize extracellular epitopes of ASCT2, thus making it impossible to analyze surface expression in live cells by flow cytometry. Analysis of membrane proteins with antibodies to intracellular epitopes presents additional difficulties, as disruption of the cell membrane to allow passage of the primary antibody can also alter the conformation of the protein or its embedment in the membrane, rendering it unrecognizable to the primary antibody. To analyze expression of ASCT2 at the protein level, B cells were activated in culture for 0, 12, and

24 hours and then fixed with para-formaldehyde followed by gentle overnight permeabilization in 0.1% saponin buffer. This method enabled me to accurately analyze ASCT2 expression at the protein level using flow cytometry. Time-dependent upregulation of ASCT2 up to 24 hours was also observed at the protein level (Figure 15). These results establish that ASCT2 expression is induced during the first 24 hours after BCR ligation.

Specific inhibition of ASCT2 mediated transport by the L-glutamine analog GPNA inhibits activation in a manner similar to that observed under L-glutamine depletion

Having confirmed that ASCT2 is upregulated following B cell activation, I set out to determine whether inhibition of ASCT2 would induce aberrant activation similar to those observed in L-glutamine depleted conditions. A glutamine analog, L-γglutamyl-p-nitroanilide (GPNA) has been shown to act as a specific inhibitor of ASCT2 mediated transport at millimolar concentrations.²²³ Small dense B cells were activated with anti-Ig in the presence or absence of 5 mM GPNA for 24 hours and analyzed for several activation parameters previously determined to be affected or not affected by a lack of extracellular glutamine. Surface CD86 expression, which had been observed to increase in activated B cells regardless of glutamine availability, was not measurably affected by GPNA treatment (Figure 16). I observed low surface CD71 expression at levels similar to that of naïve B cells in cells treated with GPNA, just as had previously been observed in glutamine deprived cells (Figure 17). Total intracellular protein content increase was also diminished in anti-Ig stimulated B cells treated with GPNA (Figure 18). Finally, GPNA treated B cells exhibited impaired cell size increase similar to that observed in glutamine deprived cells as measured by Forward Scatter (Figure 19a) and cell diameter (Figure 19b). Collectively, these results suggest that ASCT2 inhibition by GPNA is sufficient to produce activation defects parallel to those observed due to lack of extracellular glutamine, indicating that ASCT2 is a critical mediator of glutamine uptake following B cell receptor ligation.

Extracellular L-leucine supplementation does not restore activation events in Lglutamine depleted B cells

Studies in cancer cell lines and T cells have uncovered a mechanism by which ASCT2 and LAT1 act in partnership to coordinate glutamine uptake and leucine uptake.^{162,164,165} In these studies, supplementation of the cell culture media with varying concentrations of leucine was able to reverse defects in autophagy and differentiation events in both GPNA treated cells or ASCT2 / LAT1 knockdowns or knockout mice.^{162,164} It is possible that the importance of ASCT2 activity during B cell activation is wholly or partly due to partnership with LAT1 for leucine uptake. To determine whether this mechanism regulates B cell activation, B cells were treated with 5 mM GPNA or alternatively cultured in glutamine depleted media and supplemented with 4 mM or 7 mM extracellular leucine. Cells were then analyzed for several activation parameters, including surface CD71 expression and cell size increase, and compared to cells activated in complete media. Interestingly, although a marginal increase in cell size and CD71 expression was observed when GPNA treated cells were supplemented with extracellular leucine, no measurable rescue effect was observed when cells cultured in the absence of glutamine were supplemented with high extracellular leucine (Figure 20).

Similar results were obtained when cell size was examined by mean forward scatter; extracellular leucine did not rescue activation induced cell size increase during either GPNA treatment or glutamine depletion (Figure 21).

Supplementation with a cell permeable form of α -ketoglutarate is able to rescue activation events in cells cultured without L-glutamine

Having determined that leucine supplementation failed to measurably restore activation effects in the absence of extracellular glutamine, it was evident that glutamine was providing a role other than to increase leucine import. As described, glutamine has been established as an important metabolic substrate in several proliferating cell types, and is capable of feeding into several metabolic pathways. The first two steps of glutamine metabolism serve to generate α -KG, and it is from here that glutaminederived carbon is able to enter the TCA cycle. I therefore hypothesized that the production of α -KG from glutamine could be an essential function of glutamine during B cell activation, to allow glutamine to be used for ATP production or biosynthetic To investigate the significance of glutamine-derived α -KG on B cell reactions. activation processes, a cell permeable α -KG analog was used, di-methyl 2-oxoglutarate (for the purposes of this work referred to as simply α -KG).^{173,224,225} Naïve B cells were cultured in complete media, media lacking glutamine supplemented with 6.9 mM dimethyl- α -KG, or complete media containing 6-diazo-50xo-L-norleucine (DON), an inhibitor of glutaminases.^{226,227} Initially, supplementation with the cell permeable α -KG analog was highly cytotoxic, most likely due to the associated pH decrease in the media

following its addition (pH ~6.95). To combat this effect, cell culture media was readjusted to physiological pH 7.4 following the addition of α -KG, and filter sterilized. Supplementation of α -KG restored both cell size increase (Figure 22) and CD71 expression (Figure 23) in the absence of extracellular glutamine, while DON treatment inhibited these events.

mTORC1 activation depends on glutamine transport through ASCT2, as well as subsequent glutaminolysis

Previous studies have shown that α -KG is required for mTORC1 activation by a mechanism that involves RAG GTPase recruitment to the lysosome.²⁰⁴ Interestingly, this pathway is necessary for T cell differentiation into effector subsets.^{136,190,195,196} Since mTORC1 is a mediator of cell growth and many of the activation defects observed in B cells in the absence of glutamine are growth related, we examined whether glutamine derived α -KG is required for mTORC1 activity. For these studies, we monitored phosphorylation of S6, a downstream target of mTORC1, to measure mTORC1 activity. S6 is an ideal target to assay mTORC1 activity as it will only be phosphorylated downstream of mTORC1, and allows strict confirmation that mTORC1 has been activated.¹⁹² S6-pS235/236 was analyzed by flow cytometry and western blot in B cells under several different culture conditions shown (Figure 24a, 24b). S6 phosphorylation increased in B cells activated for 24 hours with anti-Ig as compared to naïve B cells, which had very low levels of positive staining. Glucose depletion had no effect on mTORC1 activity, however glutamine depletion markedly decreased levels of mTORC1

activity. In contrast to recent work in T lymphocytes showing that supplementation with high extracellular levels of leucine rescued mTORC1 activity in an ASCT2 knockout mouse model, we found that leucine supplementation had no such rescuing effect in the absence of extracellular glutamine.¹⁶² Supplementation with extracellular α -KG was able to restore mTORC1 activity levels. Additionally, treatment with GPNA or DON inhibited mTORC1 activity to an extent similar to that observed in the absence of glutamine. These findings indicate that glutaminolysis derived α -KG is crucial for mTORC1 activity. 5' AMP-activated protein kinase (AMPK) is an upstream regulator of mTORC1 that inactivates mTORC1 in response to decreased cellular ATP levels.^{129,155} AMPK classically becomes activated when glucose levels are low, and inhibits mTORC1 to decrease energy consuming reactions until ATP levels have been restored.^{153,154} Because AMPK is regulated by nutrient availability, I investigated whether it was activated in B cells in response to glucose or glutamine depletion. Due to very high background staining when analyzing the phosphorylated, active form of AMPK by western blot, it is not possible to definitely state whether nutrient limitation has an activating effect. However, p-AMPK levels do not appear to be significantly increased in glucose depleted cells (Figure 25), correlating with my finding that glucose depletion does not inhibit mTORC1 activity.

α -ketoglutarate is not required for histone or DNA demethylation during B cell activation

In addition to serving as a metabolic substrate in the TCA cycle, α -KG serves as a

required substrate for epigenetic remodeling enzymes TET and JHDM, responsible for DNA and histone demethylation respectively.¹⁷⁹⁻¹⁸¹ To determine whether glutamine derived α -KG contributes epigenetic remodeling during B cell activation, the cell permeable α -KG analog was used. Additionally, D- α -hydroxyglutaric acid (2HG), an analog of α -KG that cannot be utilized as a substrate by TET or JHDM was used as a negative control.^{184,185} To determine the effect of α -KG on DNA demethylation during B cell activation, global levels of 5-methylcytosine (5mC) were analyzed by flow cytometry (Figure 26). No significant difference was observed between 5mC levels in quiescent cells and cells activated for 24 hours either in complete media, 2HG treated, or glutamine deprived and α -KG supplemented. Although decreased 5mC levels were observed in glutamine deprived activated cells, this is possibly an effect of increased sub-G₁ DNA content, since no other α -KG dependent changes in 5mC were observed. To examine whether α -KG is essential for histone demethylation, we examined levels of Histone 3 lysine 9 (H3K9) tri-methylation by western blot as compared to total Histone 3 (H3) levels (Figure 27). In fact, H3K9 Me3 was found to increase after 24 hours of anti-Ig stimulation, and this increase was independent of glutamine or α -KG availability. Although site-specific demethylation cannot be ruled out, this data indicates that glutamine derived α -KG is not required for global epigenetic remodeling by TET or JHDM enzymes.

FIGURES AND LEGENDS

Figure 12. Glutamine uptake in activated B cells is primarily sodium-dependent. B cells were stimulated with 300ng/mL PMA plus 400ng/mL ionomycin for 18 hours, then washed and resuspended in choline-KR buffer for 30 min. Transport was initiated by mixing cells with 200uL of choline-KR buffer with 4uCi/mL [³H] glutamine in the presence or absence of sodium. Cells were separated by centrifugation through a bromodecane and 20% perchloric acid gradient. The perchloric acid layer was quantified using liquid scintillation spectrophotometry. Data is expressed as CPM/10⁶ cells/sec, sodium dependent uptake is shown in black and sodium independent uptake is shown in grey. Each time point represents 3 independent uptake measurements. Experiment performed by CA Doughty, unpublished data.

Figure 12.



Figure 13. System ASC activity mediates glutamine uptake in B cells. Glutamine transport assay was performed as in (Figure 12) using [³H] glutamine alone, [³H] glutamine plus 1mM leucine (Leu), or [³H] glutamine plus 1 mM threonine (Thr), in the presence or absence of sodium, with or without 1 mM methylaminoisobutryic acid (meth-AIB). After 90 seconds, both sodium dependent and sodium independent uptake were performed in quadruplicate; the graph shows average sodium dependent uptake with average sodium independent uptake values subtracted, expressed as CPM/10⁶ cells/90 sec. Experiment performed by CA Doughty, unpublished data.

Figure 13.



Figure 14. ASCT2 mRNA is upregulated in response to BCR cross-linking. Naïve B cells were cultured complete media and stimulated with 10 μ g/mL anti-Ig, and harvested at the indicated time points. cDNA was prepared using MACS One-step cDNA Isolation Kit, and analyzed by qPCR for ASCT2 expression. Expression was normalized using β 2M as a control as described in the Materials and Methods. Data is expressed as relative expression, and results are representative of 3 independent experiments. Error bars represent standard deviation from the mean of relative expression.

Figure 14.



Figure 15. ASCT2 protein is upregulated during B cell activation. Naïve B cells were cultured in complete media and stimulated with 10 μg/mL anti-Ig for the indicated times, and compared to naïve cells. Cells were collected and stained for ASCT2 expression after fixation and permeabilization as described in the Materials and Methods. Cells were analyzed by flow cytometry; results are representative of 3 independent experiments; error bars represent standard deviation from the mean. Data is expressed as Mean Fluorescence Intensity (MFI).

Figure 15.



ASCT2 Protein Levels

Figure 16. Inhibition of ASCT2 mediated transport by GPNA does not impair

CD86 upregulation. Naïve B cells were activated with 10 µg/mL anti-Ig and cultured in either complete (nutrient replete) media or complete media containing 5mM L- γ glutamyl-p-nitroanilide (GPNA) for 24 hours and compared to naïve cells. Cells were collected and then analyzed by flow cytometry for surface expression of CD86. Data represents analysis of live gated B cells and is expressed as the percentage of cells positively stained for expression of CD86. The data are representative of 3 independent experiments. Error bars represent standard deviation from the mean. Shown below are representative histograms of CD86 expression for cells cultured in complete media (red) or complete media plus 5 mM GPNA (green), overlaid with expression on naïve cells (grey).

Figure 16.





Figure 17. Inhibition of ASCT2 mediated transport by GPNA impairs CD71

upregulation. Naïve B cells were activated with 10 μg/mL anti-Ig and cultured in either complete (nutrient replete) media or complete media containing 5mM L-γ-glutamyl-p-nitroanilide (GPNA) for 24 hours and compared to naïve cells. Cells were collected and then analyzed by flow cytometry for surface expression of CD71. Data represents analysis of live gated B cells and is expressed as Mean Fluorescence Intensity (MFI). The data are representative of 3 independent experiments. Error bars represent standard deviation from the mean. Shown below are representative histograms of CD71 expression for cells cultured in complete media (red) or complete media plus 5 mM GPNA (green), overlaid with expression on naïve cells (grey).

Figure 17.





Figure 18. Inhibition of ASCT2 mediated transport by GPNA impairs increased intracellular protein content. Naïve B cells were activated with 10 μ g/mL anti-Ig and cultured in either complete (nutrient replete) media or complete media containing 5mM L- γ -glutamyl-p-nitroanilide (GPNA) for 24 hours and compared to naïve cells. Cells were collected and stained with FITC as described in the Materials and Methods, and analyzed by flow cytometry. Data is expressed as Mean Fluorescence Intensity (MFI). Results are representative of 3 independent experiments. Error bars represent standard deviation from the mean.





Figure 19. Inhibition of ASCT2 mediated transport by GPNA impairs cell size increase following activation. B cells were activated with 10 μ g/mL anti-Ig and cultured in either complete media or complete media containing 5mM L- γ -glutamyl-p-nitroanilide (GPNA) for 24 hours and compared to naïve cells. *A*. Cells were analyzed by flow cytometry for forward scatter, an indicator of cell size, gated on live cells. Data is expressed as Mean Forward Scatter. Results are representative of 3 independent experiments.. Error bars represent standard deviation from the mean. *B*. Cells were analyzed after 24 hours for mean cell diameter by the Millipore ScepterTM. Data is expressed as mean cell diameter (microns). Naïve cells are shown in blue, complete media shown in red, GPNA treated shown in green. Results are representative of 3 independent experiments.

Figure 19.





B

Cell Diameter (um)



Figure 20. Extracellular L-leucine supplementation does not rescue CD71 expression in the absence of L-glutamine. Naïve B cells were activated with 10 µg/mL anti-Ig and cultured in either complete media, media lacking L-glutamine, media lacking L-glutamine supplemented with 4mM or 10mM L-leucine, complete media containing 5mM GPNA, or complete media containing 5mM GPNA supplemented with 4mM or 10mM L-leucine for 24 hours. Cells were analyzed by flow cytometry for surface expression of CD71, gated on live cells. Data is expressed as Mean Fluorescence Intensity. Results are representative of 3 independent experiments. Error bars represent standard deviation from the mean. Representative histograms of CD71 expression are shown below; legend indicates lines used to represent culture conditions.
Figure 20.



Figure 21. Extracellular L-leucine supplementation does not rescue cell size. B cells were activated with 10 μg/mL anti-Ig and cultured in either complete media, media lacking L-glutamine, media lacking L-glutamine supplemented with 4mM or 10mM L-leucine, complete media containing 5mM GPNA, or complete media containing 5mM GPNA supplemented with 4mM or 10mM L-leucine for 24 hours. Cells were analyzed by flow cytometry for forward scatter, an indicator of cell size, gated on live cells. Data is expressed as Mean Forward Scatter (Mean FSC). Results are representative of 3 independent experiments. Error bars represent standard deviation from the mean. Representative histograms of CD71 expression are shown below; legend indicates lines used to represent culture conditions.

Figure 21.



Figure 22. Glutaminolysis contributes to increased cell size. Naïve B cells were activated with 10 μ g/mL anti-Ig and cultured in either complete media, media lacking L-glutamine supplemented with 6.9mM α -KG, or complete media containing 40 μ M DON for 48 hours and compared to naïve cells. Cells were analyzed by flow cytometry for forward scatter, gated on live cells. Data is expressed as Median Forward Scatter (Median FSC), results are representative of two independent experiments. Error bars represent standard deviation from the mean.





Figure 23. Glutaminolysis contributes to increased CD71 expression. Naïve B cells were activated with 10 μ g/mL anti-Ig and cultured in either complete media, media lacking L-glutamine supplemented with 6.9mM α -KG, or complete media containing 40 μ M DON for 48 hours and compared to naïve cells. Cells were analyzed by flow cytometry for surface expression of CD71, gated on live cells. Data is expressed as Mean Fluorescence Intensity. Results are representative of 3 independent experiments. Error bars represent standard deviation from the mean.





Figure 24. Glutaminolysis is essential for mTORC1 activity. Naïve B cells were activated with 10 µg/mL anti-Ig and cultured in either complete media, media lacking glucose, media lacking L-glutamine, media lacking L-glutamine supplemented with 4 mM L-leucine, media lacking L-glutamine supplemented with 6.9 mM α -KG, complete media containing 5 mM GPNA, complete media containing 40 µM 6-diazo-5-oxo-Lnorleucine (DON), or complete media containing 20 nM rapamycin for 24 hours and compared to naïve cells. *A*. Cells were fixed and permeabilized as described in the Materials and Methods and analyzed by flow cytometry for intracellular expression of S6-pSer235/236 to determine activity of mTORC1. Data is expressed as the mean percentage of cells that stained positively for p-S6 of 3 independent experiments, error bars represent standard deviation from the mean. A minimum of 5000 cells were counted in each sample/condition. *B*. Cellular lysates were prepared in Triton-X 100 buffer after 24 hours in culture. Equivalent amounts of total protein were analyzed for expression of S6-pSer235/236 or total S6 by western blot as described in the Materials and Methods.

Figure 24.







Figure 25. AMPK phosphorylation is not clearly affected by glucose availability when analyzed by western blot. Naïve B cells were activated with 10 μ g/mL anti-Ig and cultured in either complete media, media lacking glucose, media lacking Lglutamine, media lacking L-glutamine supplemented with 4 mM L-leucine, or media lacking L-glutamine supplemented with 6.9 mM α -KG for 24 hours and compared to naïve cells. Cellular lysates were prepared in Triton-X 100 buffer after 24 hours in culture. Equivalent amounts of total protein were analyzed for expression of S6pSer235/236 or total S6 by western blot as described in the Materials and Methods.



Figure 26. FACS analysis of global DNA demethylation does not indicate a contribution of glutaminolysis. B cells were activated with 10 μ g/mL anti-Ig and cultured in either complete media, media lacking glucose, media lacking L-glutamine, media lacking L-glutamine supplemented with 4mM L-leucine, media lacking L-glutamine supplemented with 6.9 mM α -KG, or complete media containing 5 mM 2HG for 24 hours and compared to naïve cells. Live cells were sorted by PI exclusion and purified using BD FACSAria cell sorter. Cells were then fixed, permeabilized, and DNase treated using the BD BrdU kit according to the manufacturers instructions. Cells were stained using an anti-5-methylcytosine antibody and analyzed by flow cytometry. Data is expressed as Mean Fluorescence Intensity (MFI). Results are representative of 2 independent experiments. Error bars represent standard deviation from the mean.





Figure 27. Global histone demethylation is not measurably affected by

glutaminolysis during B cell activation. Naïve B cells were activated with 10 μ g/mL anti-Ig and cultured in either complete media, media lacking glucose, media lacking L-glutamine, media lacking L-glutamine supplemented with 4mM L-leucine, media lacking L-glutamine supplemented with 6.9mM α -KG, or complete media containing 5mM 2HG for 24 hours and compared to naïve cells. Cellular lysates were prepared in RIPA buffer after 24 hours in culture. Equivalent amounts of total protein were analyzed for expression of tri-methyl H3K9 or total Histone 3 by western blot, as described in the Materials and Methods. Results are representative of 2 independent experiments.

Figure 27.



CHAPTER FIVE

Nutrients regulate B cell functions and differentiation

RESULTS

Glut1 upregulation occurs in a L-glutamine dependent manner following BCR crosslinking. mTORC1 activity and glutaminolysis are partly necessary for activation induced Glut1 upregulation.

Glut1 is the primary mediator of glucose transport in B lymphocytes, and while expressed at low levels in quiescent cells it is upregulated in response to B cell stimulation.^{131,132,134} In work previously published, the Chiles lab demonstrated that mTORC1 inhibition caused a moderate decrease in glucose uptake and glycolysis. This work also established that inhibition of PI3K signaling had a more complete inhibitory effect on glucose uptake and metabolism in B cells.¹³¹ Although mTORC1 activity is only partly required for glucose metabolism, I sought to investigate whether glutamine availability could act through mTORC1 signaling to promote glucose transporter expression, as has been suggested by studies in other cell types.^{228,229} To determine the role of glutamine mediated mTORC1 activity in Glut1 upregulation, I analyzed Glut1 expression by flow cytometry. As previously described, Glut1 was upregulated in B cells activated with anti-Ig in complete media as compared to naïve cells, and this upregulation occurred in a time dependent manner. Interestingly, glucose depletion prompted a slight increase in Glut1 induction as compared to cells activated in complete media, while L-glutamine depletion abrogated this time-dependent upregulation (Figure 28). L-glutamine depletion also appeared to inhibit Glut1 expression at later time points, in line with our results indicating that very early activation events up to 12 hours following BCR crosslinking can proceed in the absence of L-glutamine. To further characterize the effects of L-glutamine limiting conditions, we analyzed Glut1 expression after 24 hour anti-Ig stimulation in conjunction with inhibitors of L-glutamine uptake (GPNA), or mTORC1 activity (rapamycin). L-Glutamine depletion and GPNA treatment both inhibited Glut1 upregulation, with expression levels observed similar to that in naïve cells. This finding indicates that L-glutamine uptake through the transporter ASCT2 is essential for maximal induction of Glut1 during B cell activation. Rapamycin treatment resulted in expression levels below that observed in naïve cells (Figure 29). When taken together with our finding that L-glutamine availability is required for mTORC1 activity, this result indicates that glutamine availability acts through regulation of mTORC1 activity to affect Glut1 expression levels.

mTORC activity and L-glutamine availability are required for increased glucose uptake following B cell activation

To determine the functional significance of decreased Glut1 expression in activated B cells, I measured glucose uptake using a cell permeable fluorescent analog of glucose, 2NBDG.^{230,231} Glucose uptake was increased in anti-Ig stimulated B cells as compared to naïve cells, and was actually increased in glutamine starved and rapamycin treated cells, however not to the maximal extent observed in complete media (Figure 30). This implies there is a functional significance of decreased Glut1 expression in the absence of glutamine or mTORC1 activity that can be correlated to decreased glucose uptake.

ATP citrate lyase (ACLY) activity is required to support endomembrane expansion

during B cell activation

Additional work in the Chiles lab has focused on investigating the role of glucose metabolism in support of *de novo* lipid synthesis during B cell activation. It is important to note that to examine B cell differentiation events that occur later than cell cycle entry, such as Ig production and CD138 expression, naïve B cells require LPS to induce differentiation into plasma Ig secreting cells.^{74,232} Stimulation with anti-Ig is only sufficient to induce early signaling events, cell growth, and S phase entry, however does not provide the necessary stimulus for differentiation events. For this reason, LPS was utilized as the stimulant for experiments looking at endomembrane expansion and induction of plasma cell markers (e.g., CD138, Blimp1), as this method of stimulation is capable of inducing differentiation of naïve B cells within several days. Our previous work suggested that ACLY activity is required for glucose-dependent *de novo* synthesis of neutral and acidic lipids, including phosphatidylcholine (PC) and ceramide (CM); both of these lipids are required for endoplasmic reticulum (ER) expansion in B lymphocytes.^{133,233} It therefore seemed likely that ACLY activity might contribute to expansion of the endomembrane secretory network in response to LPS stimulation. To assess the expansion of the ER compartment, I stained ex vivo B lymphocytes with ER-Tracker[™] Green, an ER-specific probe. Intracellular staining of B cells cultured in the absence of LPS revealed a small area of staining that expanded substantially following stimulation with LPS for 24 h and 48 h (Figure 31b). Treatment of LPS-stimulated B cells with C-9 or Medica 16, two specific ACLY inhibitors with distinct mechanisms of action, resulted in decreased and/or altered intracellular staining. Flow cytometry of ER-Tracker[™] stained B cells also revealed a significant reduction in mean fluorescence intensity in LPS-stimulated B cells treated with C-9 (Figure 31a). For these experiments, the viable B cell population was gated and subsequently analyzed for fluorescence intensity by flow cytometry.

ACLY activity is required for LPS induced plasma-like phenotypic changes

To investigate further the involvement of ACLY activity in plasma-like B cell differentiation, I evaluated whether ACLY activity was required for LPS-induced surface CD138 (Syndecan-1) expression in splenic B lymphocytes; CD138 is expressed upon B cell differentiation into plasmablasts and plasma cells.^{234,235} LPS stimulation of splenic B cells increased surface CD138 expression (Figure 32). To minimize the confounding effects of decreased viability at later time points observed with C-9, B cells were instead treated with Medica 16, a small molecule inhibitor of ACLY that is structurally unrelated to C-9 and has been used both *in vitro* and *in vivo*. Treatment of LPS-stimulated B cells with Medica 16 resulted in a significant reduction in CD138 expression of Blimp-1, which is required for development of immunoglobulin-secreting cells and for maintenance of long lived plasma cells.²³⁶ I found that LPS-induced Blimp-1 expression was reduced in splenic B cell cultures incubated with Medica 16 (Figure 33).

Nutrients are not required for antigen-receptor endocytosis and trafficking

In order to further determine functional relevance of nutrient availability, I sought to investigate the effects of glucose or glutamine limiting conditions on antigen

presentation by B cells. In order to present antigen on MHCII, B cells must first endocytose bound BCR-antigen complex and transport it to the lysosome, where antigen will undergo processing and MHCII loading to be transported back to the cell surface.^{76,237,238} The lysosome is also the site of mTORC activation, and I hypothesized that perhaps lysosomal localization is negatively impacted in the absence of glutamine or glucose.²⁰⁴ An established technique for examining antigen-receptor endocytosis and transport to the lysosome by microscopy has been described in the literature.²³⁷ Using this technique and in collaboration with Mary Ann Collins in the Folker laboratory, I examined colocalization of BCR and the lysosomal associated membrane protein (LAMP-1) at 0, 15, and 60 minutes following BCR crosslinking, and colocalization can typically be observed within 30-60 minutes.²³⁷ My findings indicate that BCR endocytosis and trafficking occurs independently of either glucose or glutamine availability (Figure 34).

Glutamine uptake contributes to LPS induced differentiation in IL-10 secreting regulatory B cells

Several recent studies have demonstrated that in T cells, limited glutamine availability or ASCT2 knockout led to increased differentiation into regulatory T cell subsets, with concurrent decreased differentiation into pro-inflammatory subsets.^{162,191,196} To investigate whether glutamine availability similarly regulated differentiation into B cells, I chose to focus on differentiation into the IL-10 secreting regulatory B cell subset, so called B10 cells.^{108,110} These cells have been shown to play

important roles in autoimmune disease states, and are negative regulators of T cell proinflammatory responses.^{115,116} B10 cell differentiation from naïve splenic B cells can be induced in culture by LPS stimulation, followed by PMA and ionomycin treatment. LPS stimulation times of up to 48 hours, followed by 5 hours of PMA and ionomycin treatment have been shown to maximally increase B10 differentiation, with about 8% of cells exhibiting an IL-10 secretion phenotype.²⁰⁷ To study the role of glutamine in regulating B10 differentiation, naïve splenic B cells were stimulated with LPS and cultured in complete media, or media lacking glutamine for 48 hours. During the final 5 hours of culture, cells were treated with the addition of PMA and ionomycin to induce differentiation, and monensin to inhibit protein transport and retain any secreted IL-10, for intracellular staining, as described in the literature.²⁰⁷ LPS stimulation in the absence of extracellular glutamine resulted in a near complete block in intracellular IL-10 accumulation as determined by flow cytometry (Figure 35). Additionally, IL-10 secretion was investigated by ELISA to quantitate concentrations of IL-10 in cell culture supernatants of B cells stimulated with LPS for 72 hours (Figure 36). Glutamine depletion resulted in decreased IL-10 secretion, as did GPNA treatment, as compared to cells stimulated in complete media. These results indicate that ASCT2 mediated glutamine uptake contributes to LPS induced B10 differentiation in vitro.

FIGURES AND LEGENDS

Figure 28. Upregulation of Glut1 occurs in a time dependent manner and depends on extracellular L-glutamine availability. Naïve B cells were activated with 10 µg/mL anti-Ig and cultured in either complete media (Dark Blue), media lacking glucose (Green), or media lacking L-glutamine (Light Blue) for 6, 12, or 24 hours and compared to naïve cells (white). Cells were collected then fixed and permeabilized and analyzed by flow cytometry for expression of Glut1 as described in the Materials and Methods. Data is expressed as Mean Fluorescence Intensity (MFI). Results are representative of 2 independent experiments. Error bars represent standard deviation from the mean.

Figure 28.



Glut1 Timecourse

Figure 29. Upregulation of Glut1 depends on glutamine uptake and mTORC activity. Naïve B cells were activated with 10 μ g/mL anti-Ig and cultured in either complete media, media lacking glucose, media lacking L-glutamine, complete media containing 5mM GPNA, or complete media containing 20 nM rapamycin for 24 hours and compared to naïve cells. Cells were fixed and permeabilized and analyzed by flow cytometry for expression of Glut1 as described in the Materials and Methods. Data is expressed as Mean Fluorescence Intensity (MFI). Results are representative of 2 independent experiments. Error bars represent standard deviation from the mean.

Figure 29.



Figure 30. Glut1 expression levels correlate with glucose uptake. Naïve B cells were activated with 10 μ g/mL anti-Ig and cultured in either complete media, media lacking L-glutamine, or complete media containing 20nM rapamycin for 24 hours and compared to naïve cells. Cells were harvested after 24 hours in culture, washed, and incubated in media lacking glucose for 4 hours. Cells were then incubated in culture medium containing 150 μ g/mL 2-NBDG for 30 minutes and analyzed by flow cytometry, gated on live cells. Data is expressed as Mean Fluorescence Intensity (MFI). Results are representative of 3 independent experiments. Error bars represent standard deviation from the mean.

Figure 30.



Figure 31. ACLY activity is required for increased endoplasmic reticulum

membrane expansion in LPS-stimulated B lymphocytes. *A*. B cells were cultured in medium alone (*Media*) or stimulated with 50 µg/ml LPS. Parallel LPS-stimulated B cells were treated with 50 µM compound-9 (*LPS* + *C9*) for 48 h. Cells were collected, and ER content was assessed by staining with ER-TrackerTM and flow cytometry. The data are expressed as mean fluorescence intensity (*MFI*). *Error bars*, S.D. of two independent measurements. *B*. B cells were cultured in medium alone or stimulated with 50 µg/ml LPS. Parallel LPS-stimulated B cells were treated with 50 µM compound-9 (*C9*) or 40 µM Medica 16 for the indicated times. Cells were then collected and stained with ER-TrackerTM in order to assess ER content and fluorescence microscopy as described under "Experimental Procedures."

Figure 31.

A



ER Tracker

e 0

24 hour

48 hour



B

Figure 32. ACLY activity is required for LPS-induced CD138 upregulation in B

lymphocytes. B cells were cultured in medium alone or stimulated with 50 μg/ml LPS for 72 h; parallel LPS-stimulated B cells were treated with 40 μM Medica 16 (*LPS+Medica 16*). Cells were then evaluated for the expression of surface CD138 expression by flow cytometry following gating on the viable cell population. The data are expressed as mean fluorescence intensity (*MFI*). *Error bars*, S.D. of two independent measurements.

Figure 32.



Figure 33. ACLY activity is required for LPS-induced Blimp-1 induction in B

lymphocytes. B cells were cultured in medium alone or stimulated with 50 μg/ml LPS for 72 h; parallel LPS-stimulated B cells were treated with 40 μM Medica 16 (*LPS+Medica 16*). Cells were evaluated for the expression of Blimp-1 by flow cytometry following gating on the live cell population. The data are represented as percentage of Blimp-1⁺ B cells. *Error bars*, S.D. of two independent measurements.

Figure 33.



Figure 34. Nutrients are not required for early antigen-receptor endocytosis and

trafficking. Cellular localization of BCR (green) and LAMP-1 (red) in purified, naïve B cells 0-60 minutes following BCR crosslinking in either complete media (Comp), media lacking glutamine, or media lacking glucose. 10⁶ Naïve B cells were stained and analyzed by confocal microscopy as described in the Materials and Methods. Experiments were performed in duplicate; images are representative of the overall cell population.
Figure 34.

_	Comp			— Glutamine			— Glucose		
	lgM	LAMP-1	Merge	lgM	LAMP-1	Merge	lgM	LAMP-1	Merge
0'	0			0	C	0	\bigcirc	Q	0
15'	6		٢	Ŕ	â.	•		6	0
60'	d.	¢.	۴	44	ŧ	¢	-89	4	١

Figure 35. ASCT2 mediated glutamine uptake is required for LPS induced B10 differentiation. Naïve B cells were cultured in complete media or media lacking glutamine and stimulated with 10 μg/ml LPS for 48 hours, treated with 100 nM PMA, 500 nM ionomycin, and 2 μM monensin for the final 5 hours. Cells were fixed and permeabilized and evaluated for cytoplasmic IL-10 by flow cytometry as described in the Materials and Methods. Data is shown as a representative plot of cell size vs. IL-10 fluorescence, gated on the percentage of IL-10 positive cells. Results are representative of 3 independent experiments.

Figure 35.



Figure 36. ASCT2 mediated glutamine uptake is required for LPS induced IL-10 secretion. Naïve B cells were cultured in complete media, media lacking glutamine, or complete media containing 5 mM GPNA and stimulated with 10 μ g/ml LPS for 72 hours and compared to naïve cells. Cell culture supernatants were collected and IL-10 concentrations were determined by ELISA. Data is presented as the average IL-10 concentration of three replicates, error bars display standard deviation from the mean.

Figure 36.



CHAPTER SIX

Discussion

The regulation of nutrient uptake and metabolism to support growth and proliferation during B cell activation remains a poorly understood area of research. Early studies have established several decades past that mixed lymphocyte populations. following antigen recognition, engage in increased glucose and glutamine consumption and upregulate metabolic enzymes to process these nutrients.^{42,149,211} This increased nutrient consumption supports cellular growth and proliferation, as well as provides energy in the form of ATP to fuel cellular processes. Although in recent years, much work has been accomplished to address these questions specifically in T cells, comparatively few studies have been conducted to address nutrient utilization during the B cell response. Considering the substantial functional differences between the T cell mediated immune response and the B cell mediated humoral immune response, it is reasonable to conceive that the regulation of B cell metabolism during the activation response may significantly differ from that of T cells. In support of this theory, work has shown that following receptor crosslinking, T cells increase glycolysis to a greater extant from the resting state than B cells with a comparative decrease in oxidative phosphorylation (OXPHOS), while B cells utilize glycolysis and OXPHOS to similar extents.¹³⁴ Since so little is known about how B cells utilize nutrients to fuel growth and proliferation, one of the aims of this study was to shed new light on this process.

My research suggests that both extracellular L-glutamine and glucose are dispensable for very early activation events following BCR crosslinking, as demonstrated by unperturbed expression of CD86. However, later growth related events including CD71 upregulation, cell size increase, and protein and DNA synthesis are strictly dependent on the availability of extracellular L-glutamine. In contrast, extracellular glucose is only partially required for these later activation events to occur, with glucose depleted B cells displaying slightly inhibited growth related responses. To fuel these growth related events, glutamine uptake increases rapidly following BCR crosslinking and in a sodium-dependent and system ASC dependent manner. The high affinity L-glutamine importer, ASCT2, is upregulated following B cell activation. Inhibition of ASCT2 mediated transport by GPNA treatment caused similar activation defects to those observed during L-glutamine starvation, highlighting the essential nature of ASCT2 mediated glutamine import. Interestingly, in contrast to studies in T-cells, Lleucine supplementation provided limited to no rescuing effect on these defects, indicating that L-glutamine or its metabolic products are essential.¹⁶² Indeed, we found that supplementation with a cell permeable form of α -KG was able to restore activation events, including mTORC1 activity. Inhibition of the glutaminolytic enzyme glutaminase by DON also resulted in decreased mTORC1 activity and limited cell growth events, as did inhibition of ASCT2 mediated L-glutamine uptake by GPNA. mTORC1 activity is known to increase Glut1 expression at the cell surface and on this point. we observed that under conditions of L-glutamine depletion, GPNA treatment, or rapamycin treatment resulted in failure to increase BCR-induced Glut1 expression levels and glucose uptake comparably to high glutamine conditions. These findings reveal a possible nutrient sensing mechanism during B cell activation, by which mTORC1 activity senses extracellular glutamine availability via α -KG, and a lack of extracellular glutamine results in failure to undergo the growth events normally associated with B cell activation, including cell size increase, protein content. and nutrient receptors/transporters. This also provides interesting insight to the fact that the glutamine requirement precedes the glucose requirement during B cell activation, in that initial glutamine availability actually regulates the increase in glucose uptake. Together these results demonstrate a mechanism coupling the utilization of these two distinct nutrients through mTORC1 that has not previously been observed in B cells (Figure 1).



Figure 1. Model of mTORC1 coordination of nutrient uptake and metabolism. Glutamine imported through ASCT2 is metabolized to α -KG, which positively regulates mTORC1. In turn, mTORC1 contributes to Glut1 induction and glucose uptake.

Distinct glucose and glutamine requirements during early B cell activation

My initial experiments characterizing the effect of depletion of either glucose or glutamine from cell culture media reveals previously unknown roles for these two

nutrient molecules that are not interchangeable during early B cell activation. Glucose and glutamine are capable of being utilized in many of the same metabolic pathways, most notably by feeding into the TCA cycle to generate ATP or used as a carbon source for biosynthesis of macromolecules including lipids, proteins, and nucleic acids. However, their metabolism differs in several important ways, for instance, prior to entering the TCA cycle, glucose must undergo glycolysis. This process provides opportunity for glucose to be diverted from the TCA cycle such via the production of lactate from pyruvate, or through the pentose phosphate pathway.^{148,149,211} These alternate pathways of glucose utilization allow glucose derived carbon to fuel the biosynthesis of macromolecules that contribute to cell growth and division including lipids and nucleotides. Contrastingly, glutamine may only undergo two catalyzed reactions, the first steps in all metabolic reactions of glutamine, to produce α -KG.¹⁵⁸ This includes the enzyme catalyzed conversion of glutamine to glutamate, followed by conversion of glutamate to α -KG as catalyzed by GLS. α -KG can then directly enter the TCA cycle, in a much shorter pathway than glucose-derived acetyl-coA. Additionally, glutamine has no alternative ATP generating pathway such as glycolysis, making TCA cycle entry essential to the production of ATP via glutamine metabolism.¹⁵⁸ Glutamine can additionally contribute to the biosynthesis of amino acids, as well as act as an obligate nitrogen donor during nucleotide biosynthesis.¹⁴⁹ These are just a few of the distinctions between glutamine and glucose metabolism, underlining the potential for alternative roles of these two nutrients in cellular metabolism.

Stable isotope tracer labeling experiments, in which cells were cultured with

either ¹³C labeled glucose or glutamine indicate that LPS-stimulated B cells preferentially utilize glucose-derived carbon during the initial stages of activation response including the proliferative phase, but switch to a metabolic program that preferentially utilizes glutamine-derived carbon to support Ig production.¹³⁵ This study represents one of the few examples of work to investigate the differential requirement for glucose and glutamine during B lymphocyte differentiation. Although my studies found that the glutamine requirement preceded the glucose requirement, contrasting with the proposed order of nutrient utilization in the B cell stable isotope experiments, it is important to note that the stimulating agent in the experiments presented herein was anti-Ig and not LPS, which could account for the differences in our data. LPS is recognized by TLRs as opposed to the BCR and signals through distinct pathways.^{74,82,232} Additionally, the published tracer experiments were not carried out under limiting glucose or glutamine in cell culture media during activation and differentiation. It is not known whether in the absence of one or both of these nutrients the regulation of B cell activation is altered. It is also possible that early glutamine regulation of mTORC1 acts as a nutrient sensing mechanism. This mechanism has been proposed for nutrient regulation of T cell responses.^{162,206} In our B cell model, mTORC1 regulation could inhibit the cell from entering G_1 in the case of insufficient extracellular glutamine concentrations for later Ig synthesis. Our finding that low concentrations of extracellular glutamine were sufficient to restore defects associated with glutamine depletion, while glucose depletion appeared to have a concentration dependent effect is interesting in this context. If glucose is primarily metabolized during early stages of B cell activation, lower concentrations would lead to lower levels of required metabolites such as citrate required for lipid synthesis or nucleotides produced via the pentose phosphate pathway. Low levels of glutamine, conversely, could be sufficient to signal through mTORC1 that extracellular glutamine was available and to allow the cell to proceed through the glucose-metabolizing stage.

Amino acids and mTORC1 coordinate cellular metabolism in lymphocytes

Recent work has provided insight into the role of amino acid uptake and increased glycolytic metabolism in the regulation of T cell responses and the ability of mTORC1 to direct metabolic rewiring that is essential to CD4⁺ T cell differentiation.^{191,205,206,225} CD4⁺ T cells stimulated under appropriate conditions have the capacity to differentiate into $T_{\rm H}1$, $T_{\rm H}2$, $T_{\rm H}17$, or Tregs. Differentiation into $T_{\rm H}$ subsets is mTOR-dependent, with mTORC1 signaling being critical for T_H1 and T_H17 generation, while mTORC2 is required for T_{H2} .^{187,189,190} Treg differentiation is impaired by mTORC activity due to the downregulation of FoxP3 by both mTORC1 and mTORC2.^{190,195} mTORC is believed to regulate these differentiation programs through metabolic reprograming (Figure 2). mTORC1 promotes a glycolytic phenotype through increased glucose uptake and metabolism, and T_H1 and T_H17 subsets are known to be highly glycolytic while Tregs rely primarily on lipid oxidation for ATP generation.^{187,191,196,239} As discussed above, B cells are also capable of differentiation into different subsets of effector or regulatory cells following activation.¹⁰⁶ Little is known about the regulatory mechanisms that control B effector differentiation, and it remains to be addressed whether reprogramming of cellular metabolism plays a role in directing differentiation.

Importantly, inhibition of amino acid uptake by knocking out either ASCT2 or LAT1 in mice recapitulated the differentiation changes associated with mTORC1 inhibition in CD4+ T cells, exposing the ability of mTORC1 to coordinate nutrient availability and differentiation.^{162,165} Germane to my studies, it was reported that ASCT2 knockout mice contained decreased numbers of mature B cells in the spleen, indicating that ASCT2 mediated amino acid import is essential during B cell development and/or peripheral maintenance.¹⁶² For instance, ASCT2-mediated glutamine uptake may be essential during a proliferative phase of B cell development (e.g., at the large Pre-B cell stage in the bone marrow). Inhibition of proliferation during this stage of development could lead to lower total numbers of mature B cells. ASCT2 could alternatively be required for maintenance of mature B cells in the periphery by promoting cell survival or decreasing apoptosis and/or for expansion of B cells in response to antigen recognition. The results of my research indicate that GPNA treatment inhibits B-cell growth and proliferation responses by limiting ASCT2 mediated glutamine uptake and mTORC1 activity. This provides evidence to support the possibility that ASCT2 is critical for cell cycle entry in response to antigen encounter, which likely contributes to the number of splenic B cells. Interestingly, the timeline of maximum ASCT2 upregulation in B cells corresponds to the time at which negative effects of glutamine depletion are observed (24 hours). Although it is likely that many nutrient transporters in addition to ASCT2 are upregulated during these initial 24 hours as this is the time during which the B cell becomes highly transcriptionally and translationally active, it is important to note the correlation.^{186,240} It is possible that the time at which maximum glutamine import would normally occur is due to a requirement for glutamine for growth and signaling events at that specific time, and in the absence of glutamine these events can not proceed.



Figure 2. Cellular metabolism and level of mTORC activity regulate T effector differentiation, adapted from (240) (permission not required to reprint). Following TCR stimulation, CD4⁺T cells can differentiate into one of four effectors based on receipt of cytokine signals. This differentiation is also regulated by mTORC1 activity and cellular metabolism, with high mTORC1 activity and glycolysis promoting differentiation into pro-inflammatory subtypes.²³⁹

Notably, the ASCT2 knockout mouse study did not examine the effects of complete deprivation of glutamine from the cell culture media.¹⁶² Therefore, it is possible that low concentrations of glutamine were still being imported into the cell through other transporters, in a "leaky" rescue mechanism. This correlates with my results in that extracellular leucine supplementation was able to partially rescue inhibition of ASCT2 by GPNA treatment, allowing that glutamine could be carried into the cell by other transporters. However, the inability of leucine to rescue cell growth related events from complete deprivation of glutamine, along with inability to restore mTORC1 activity to any degree, indicates that glutamine is essential. Of note, I did not

culture B cells in the absence of extracellular leucine and therefore I cannot rule out the possibility that leucine availability may be also essential for cell growth and mTORC1 activity. Within the scope of my study it can be concluded that glutamine is certainly necessary, whereas leucine alone is not sufficient for mTORC1 activity and growth related B cell activation responses.

The interplay between mTOR activity and cellular metabolism has been further established in a recent study demonstrating that glucose depletion resulted in AMPK activation and inhibition of mTORC1 in CD4+ T cells.¹²⁹ AMPK negatively regulates mTORC1 in response to low intracellular ATP levels in order to limit energy consuming growth reactions until ATP levels are restored. In this study described by the Jones laboratory, an alternative energy pathway relying on glutamine metabolism and oxidative phosphorylation instead of glycolysis was utilized to promote T cell survival.¹²⁹ In my studies AMPK activity did not appear to be increased in the absence of glucose as determined by western blot, nor was mTORC1 activity diminished as shown by levels of S6 phosphorylation. In fact, the percentage of B cells with positive P-S6 staining was increased in B cells activated in the absence of glucose. My investigation also demonstrates that mTORC1 activity contributes to Glut1 upregulation and glucose uptake in B cells. Taken together, these results indicate that mTORC1 activity is likely acting primarily upstream of glucose in anti-Ig activated B cells. This represents a regulatory mechanism that is distinct from that observed in T cells.

The role of mTOR in B cells has been studied extensively, and in several previous studies, B cells have been demonstrated to be generally more sensitive to the effects of mTORC1 inhibition than T cells.^{186,241} For example, the inhibition of TCR

stimulated proliferation in T cells by rapamycin is markedly less than that in BCR stimulated B cells, with rapamycin treatment causing a near complete block in proliferation of murine B cells stimulated with anti-Ig.²⁴¹⁻²⁴³ The decreased proliferation was not correlated with decreased cell survival, indicating a mechanism distinct from autophagy or apoptosis, although the precise blocking mechanism remains unclear.²⁴² This correlates with our finding that in the absence of extracellular glutamine, activated B cells did not suffer decreased viability, providing further indication that glutamine is acting through an mTORC1-regulated mechanism. It is also known that different methods of in vitro activation of B cells (LPS vs. anti-Ig) have been shown to differentially regulate mTORC1 activity.²⁴⁴ Additionally, different subsets of mature, resting B cells have been shown to have differing basal levels of mTORC1 activity; FO B cells have low levels of basal mTORC1 activity, while MZ B cells have higher basal activity levels, and this basal activity is augmented by the addition of amino acids to cell culture media.²⁴⁴ mTORC1 and mTORC2 likely also have an important role in B cell development; an mTOR hypomorph mouse model had a partial block of development at the large pre-B to small pre-B stage, as well as decreased numbers of MZ and transitional B cells in the periphery.²⁴⁵ These key findings demonstrate the importance of mTORC signaling, and the regulation of mTORC activity in B cells, at different stages of development, indifferent B cell subsets, and in response to diverse types of stimulation. My finding that glutamine uptake and metabolism are required for mTORC1 activity following anti-Ig stimulation of naïve B cells provides a novel mechanism of mTOR regulation that was previously unknown.

Nutrients contribute to LPS induced differentiation

Unlike anti-Ig, LPS stimulation induces the proliferation and differentiation of B cells into Ig-secreting plasma cells.^{85,133,232} Prior to proliferation, the cell membrane content doubles, and during differentiation the interior of the cell undergoes a substantial morphological change.^{145,233} Expansion of the rough ER and Golgi network is induced, to cope with the demands of production and secretion of large amounts of Ig.²³⁵ Distinct changes in the ER network manifest as early as 24 h after exposure of naive B lymphocytes to LPS.^{145,233} To meet the demands of increased membrane content, B cells undertake *de novo* lipid synthesis, which can be accomplished by shuttling carbon, from nutrients such as glucose or glutamine, into acetyl-CoA production and subsequent synthesis pathways for fatty acids.²⁴⁶⁻²⁵⁰ LPS stimulation of B lymphocytes promotes an increase in ACLY enzymatic activity, the rate-limiting enzyme in the production of acetyl-CoA, as well as phosphorylation of ACLY on serine 454, which leads to increased catalytic activity.^{133,251} Previous work by the Chiles lab showed that ACLY activity is required for glucose-dependent de novo lipogenesis. This has been demonstrated by inhibition of glucose incorporation into fatty acid, cholesterol, and neutral and acidic lipids following treatment of B cells with the selective ACLY inhibitor 2-hydroxy-N-arylbenzenesulfonamide (Compound 9, C-9).¹³³ Further, this work revealed that the majority of acetyl-CoA used to support *de novo* fatty acid synthesis following LPS stimulation is derived from glucose and not glutamine. Taken together, these results point to a key role for ACLY activity in regulating glucosedependent *de novo* lipogenesis in LPS-stimulated B lymphocytes. Several of the lipids identified whose de novo synthesis is increased in response to LPS, such as phosphatidylinositol (PI), play critical roles in B lymphocyte signaling and/or differentiation.^{19,52} Additionally, synthesis of phosphatidylcholine (PC), the most abundant membrane phospholipid and the primary phospholipid in the ER compartment, was found to increase following LPS stimulation.^{233,246}

Based on these results, I investigated whether ACLY activity was necessary for ER expansion and B cell differentiation. My results showed that impaired *de novo* lipogenesis, resulting from ACLY inhibition, correlated with an inability to physically expand the ER compartment in B lymphocytes stimulated with LPS. To examine the effects of ACLY inhibition on B cell differentiation, I was able to monitor the expression of several B cell differentiation markers by flow cytometry in viable lymphocyte populations treated with the small molecule ACLY inhibitor 3,3,14,14-tetramethyl-hexadecanedioic acid (Medica 16) and/or C-9. LPS-induced surface expression of CD138, a marker for B cell differentiation, was reduced following inhibition of ACLY activity. The increased expression of Blimp-1 in LPS-stimulated splenic B cells was also reduced in the presence of Medica 16. These findings support a role for ACLY activity in B cell differentiation to a plasma-like phenotype.

A putative role for glutamine in the production of B10 regulatory cells

In order to identify the physiologic relevance for glucose and glutamine in B cells, I evaluated the impact of glucose or glutamine depletion on antigen presentation by B cells. Antigen presentation is a very early event in the response of B cells to antigen exposure and requires BCR-mediated endocytosis of antigen followed by

delivery to the lysosome, where it is then processed and complexed with MHCII and transported to the cell surface.^{76,102,238} With this in mind, I sought to initially determine if glucose or glutamine were required for BCR endocytosis and trafficking to the lysosome. Studies carried out in collaboration with the Folker laboratory revealed that in naïve, antigen-inexperienced B cells, antigen endocytosis and trafficking to the lysosome as measured by colocalization of BCR and the lysosomal marker LAMP-1 was not impacted by the availability of extracellular glucose or glutamine.²³⁷ This finding is consistent in the context of my results indicating that very early activation events prior to 24 hours following anti-Ig stimulation are not nutrient dependent. BCR endocytosis occurs rapidly following antigen encounter, and colocalization under normal conditions can be observed between 15 and 60 minutes.^{102,238} BCR endocytosis can be observed in cells that have been primed with LPS stimulation for several days, and future experiments will aim to establish whether antigen trafficking is impacted by nutrient availability in the primed B cells, as the activation in the absence of nutrients may impact lysosomal structure or endocytotic pathways at later times.

I also evaluated whether glucose or glutamine might be required for the differentiation of B cell regulatory subsets. Using an *in vitro* system developed by the Tedder laboratory to generate B10 cells from naïve splenic B cells, initially by LPS stimulation followed by PMA and ionomycin treatment, my experiments revealed that glutamine is required for LPS-induced differentiation of CD5+ CD1d^{high} IL-10 secreting regulatory B cells in *ex vivo* cultures.²⁰⁷ IL-10 secretion was found to be abolished in the absence of glutamine, as measure both by flow cytometry and ELISA. Treatment of B

cells during LPS stimulation with the ASCT2 inhibitor GPNA also blocked differentiation of B10 cells, demonstrating the importance of ASCT2 mediated glutamine uptake for differentiation. Interestingly, these findings differ to studies examining the role of glutamine in T cell differentiation, wherein glutamine depletion or ASCT2 knockout in mice resulted in increased differentiation into regulatory subsets.^{162,190,191,239} My results utilize and *in vitro* system using wild-type cells, whereas this study in CD4+ T cells examined a knockout mouse in which all tissues/cell types lack ASCT2. Therefore, my findings are cell intrinsic whereas the T cell effect could be T cell extrinsic, for instance the development of CD4+ T cells could be skewed towards a non-regulatory subtype prior to activation. B10 cells are found in mouse spleen at low frequencies (1-3%), and can be induced in vitro by diverse sets of stimuli, including LPS, CD40, and PMA plus Ionomycin.^{110,207} It is now known that a subset of progenitor B10 cells (B10pro) exists that can give rise to IL-10 secreting B cells, however this subset and their role in the generation of the total B10 cell population remains poorly understood, as do molecular mechanisms of B10 differentiation.^{207,252,253} This differentiation appears to be at least somewhat BCR dependent, however IL-10 secretion can occur in response to diverse stimuli (*i.e.*, CD40, LPS, anti-Ig).^{207,254,255} Signaling through cell surface receptors that recognize these signals drives B10 differentiation and confers the characteristic suppressive phenotype, but the exact signaling pathway is poorly understood.^{253,256-258} My results are the first to suggest a role for a nutrient molecule (*i.e.*, glutamine) in the generation of a regulatory B cell (*i.e.*, B10), and reveal an important new finding that B cell differentiation is impacted by nutrient availability. Previous work has shown that B10 differentiation is correlated with plasma-like differentiation marker CD138, and is dependent on Blimp-1.²⁵⁹ My work has also demonstrated the importance of the nutrient glucose to plasmablast differentiation, and interplay between these two nutrients could support B10 differentiation as well. A differential requirement for these two nutrients during early activation in response to anti-Ig stimulation was also demonstrated; it is possible that they support different responses during later LPS-induced differentiation events. For instance, I have demonstrated that glucose supports lipid biosynthesis and ER expansion which contributes to plasmablast differentiation, perhaps glutamine contributes to an alternative growth response such as resident ER protein synthesis, or calcium uptake.

Translational significance to human health

Although the work herein was carried out the immune system of the mouse as a model system, these results have potential applications to human health, especially when viewed in the context of other's work. For instance, it has been described that victims of burns have decreased circulating blood glutamine concentrations.^{260,261} This could be significant given that burns, being wounds, create an increased susceptibility to infection. Low glutamine levels could impair the mounting of an effective B cell response, given my demonstration that activation is severely impaired in the absence of glutamine. Recent work that has shown nutrient availability contributes to perturbations in T effector subset differentiation has led to a new interest in the role that nutrients might play in autoimmune disease.²⁰⁶ Emerging studies show that altered nutrient states exist in diseases including EAE and MS, in particular altered blood concentrations of

glutamine and glutamate.^{162,262-264} This opens the possibility that these altered nutrient states are contributing to disease state through activation or differentiation of T, or as shown in the work described herein, B cells. This is especially exciting given my findings that L-glutamine availability regulates differentiation into the regulatory B10 subset. This cell subset has been demonstrated to play a significant role in regulating T cell autoinflammatory responses.¹¹¹⁻¹¹³ Impaired B10 differentiation due to altered nutrient availability could potentially speed up or increase disease symptoms.

When considering the potential relevance to human health, it is important to note the limitations of an in vitro system. While the mouse provides a practical model system for the study of B cells, it is important to note differences in B cell biology between mice and humans. For instance, in the mouse circulating levels of B cells are proportionally much higher that that in human.^{265,266} Further, human B cells are unable to respond to LPS stimulation due to a lack of TLR4 receptors.²⁶⁷ This makes the study of glutamine contribution to LPS derived B10 cells difficult to compare to human regulatory B cells. Additionally, cells manipulated *in vitro* may not always accurately represent conditions cells would encounter *in vivo*. It is important to consider the role that other cells, such as T cells or other immunes cells that would normally be in the B cell environment, may have during B cell responses. Manipulation of isolated cells does, however, provide the only practical means to strictly control concentrations of specific nutrients that would be difficult or impossible to regulate in a living organism.

Summary and Future Directions

The results outlined in my thesis shed light on the differences in the regulation

and utilization of glucose and glutamine during the B cell activation response. Interestingly, although an absence of glutamine results in an early block at G₁ entry, with only the earliest of activation events able to proceed, glucose depletion inhibits but does not abolish activation events up to and including S phase entry during anti-Ig stimulation. Glucose is however an important contributor of carbon to *de novo* lipid synthesis for ER expansion and induction of plasma-like differentiation following proliferation under LPS stimulation. Importantly, early glutamine uptake and metabolism aids in the induction of Glut1 expression and glucose uptake in an mTORC1 dependent manner. Together, this provides an interesting potential timeline in which early glutamine uptake and metabolism helps to regulate glucose uptake that although not essential during early activation and cell cycle entry, becomes critical at later stages of the B cell response. My results also identify ASCT2 as a critical glutamine importer during these described B cell responses, and characterize a transport system for glutamine that has not previously been examined in B cells. Further, glutamine is required for differentiation into B10 cells following LPS treatment. This finding represents a heretofore-unknown regulatory role of glutamine during B cell activation, and could potentially have relevance *in vivo* in nutrient depleted microenvironments in the periphery of the immune system.



Figure 3. Timing of nutrient requirements during B cell activation and differentiation. Glutamine depletion results in as early block in cell growth responses, while glucose is essential for *de novo* lipid synthesis and plasma-like differentiation. Glutamine is also essential for IL-10 secretion at later stages of activation, although the exact timing of this block in differentiation is unknown.

Future work in this project will aim to further study the importance of ASCT2 to B cell maintenance and activation. The ASCT2 knockout mouse provides an excellent opportunity to examine the requirements for glutamine in a living system, where concentrations of glutamine may vary.¹⁶² Use of this model will enable us to also examine development of B10 cells *in vivo*, uncovering additional earlier time points during which ASCT2 is required. For instance, many B10 cells arise from the B-1 subset of B cells, and deletion of ASCT2 might inhibit the development of these cells early on, leading to a phenotype of decreased Breg populations in adult mice. Additionally, we will seek to determine the contribution of glutamine metabolism to LPS induced differentiation. Glutamine can support a variety of metabolic processes, including generation of ATP through the TCA cycle. This work has demonstrated the importance of initial steps of glutamine metabolism to support α -KG production and mTORC1 activation, however it is highly likely that glutamine is metabolized further to provide substrates for biosynthetic reactions, as well as to support energy production.

Although my studies have determined the importance of α -KG production from glutamine to support B cell responses, future experiments should aim to address the other pathways of glutamine metabolism and their role during B cell activation and differentiation. This question can be addressed using commercially available inhibitors of specific enzyme catalyzed steps in the metabolism of glutamine to determine their importance to B cell responses. Finally, there is much further investigation to be done into the requirement for glutamine during B10 differentiation. My work has established that ASCT2 mediated glutamine uptake is required to induce B10 differentiation *in vitro* following LPS stimulation, however the precise role remains to be determined. Very little is known about the molecular mechanisms regulating B10 differentiation, however a good starting point would be to determine the role of glutamine in response to different stimuli (i.e., CD40, LPS), as these stimuli have been shown to differentially induce B10 production. Additionally, glutamine metabolism could be inhibited to determine the importance of production of various glutamine derived metabolites. This could identify one or more specific metabolic programs that glutamine contributes to that are essential for B10 differentiation.

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