Isoform identification and partial characterisation of Glutaminase in CD4+ T lymphocytes

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Sam Nicholson

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LIST OF ABBREVIATIONS

ACL	ATP citrate lyase
AMP	Adenosine monophosphate
AMPK	AMP-dependant protein kinase
APC	Antigen-presenting cell
ASCT2	Neutral amino acid transporter 2
ATP	Adenosine triphosphate
BCR	B cell receptor
CaMKK2	Calcium calmodulin-dependent protein kinase
CD	Cluster of differentiation
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FAS	Fatty acid synthase
FBS	Fetal bovine serum
GAC	C-type Glutaminase
Gln	Glutamine
GLS	Glutaminase
Glu	Glutamate
GSH	Glutathione
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
ICAM	Intercellular adhesion molecule
IFNγ	Interferon-gamma
IL-x	Interleukin
KGA	Kidney-type Glutaminase
LAT	Linker for activation of T cells

LDH	Lactate dehydrogenase
LFA-1	Lymphocyte function-associated antigen 1
LGA	Liver-type Glutaminase
LKB1	Liver kinase B1
MAPK /ERK	Mitogen-activated protein kinases / Extracellular signal-regulated kinases
MDH	Malate dehydrogenase
ME	Malic enzyme
MHC	Major histocompatibility complex
mTORC	Mammalian target of rapamycin
MTX	Methotrexate
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NEAA	Non-essential amino acids
NFAT	Nuclear factor of activated T cells
NK	Natural killer cells
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered solution
PPP	Pentose phosphate pathway
PPRs	Pattern recognition receptors
PTK	Protein tyrosine kinase
qPCR	real-time Polymerase chain reaction
RA	Rheumatoid Arthritis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SNAT	Sodium-coupled neutral amino acid transporter
TCA	Tricarboxylic acid cycle
TCR	T cell receptor
ZAP70	Chain associated protein 70

ABSTRACT

The proliferating phenotype is highly glycolytic and reliant on sufficient supply and synthesis of biosynthetic intermediaries to meet the demands of rapid cell replication. Glutamine contributes to this up-regulated synthetic machinery by providing critical anaplerotic carbon to the TCA cycle; nitrogen for nucleoside production; and, through its rapid conversion to lactate, the reducing agent NADPH necessary to maintain the increase in anabolic activity. Glutaminase represents a key step in this catabolism of glutamine and has been shown to be up-regulated in many different proliferating tissues. It presents a target for the possible suppression of undesired proliferation in over-reactive immunity.

The expression of the three Glutaminase isoforms (KGA, LGA & GAC) where partially characterized using Western Blots in mature naïve and proliferating human CD4+ T lymphocytes after TCR-CD3/CD28 activation. Two Glutaminase inhibitors, BPTES and compound 968, were evaluated through ^{3}H-Thymidine incorporation assays and mass spectrometry analysis of glutamine flux. In addition, the effects of glutamine removal from proliferating populations of human CD4+ T lymphocytes were also analysed

Our results showed glutamine is absolutely necessary for optimal CD4+ T lymphocyte proliferation and that the Glutaminase isoform GAC is up-regulated in proliferating human CD4+ T lymphocytes. Its inhibition leads to attenuation in proliferative activity.

1.0 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The mammalian immune system encompasses the cells, tissues and molecules that endow the host organism with the ability to protect itself from infection and disease[1]. An immune *response* is the coordinated reaction of this system against an infection. Deficiencies in the immune system can quickly lead to serious illness and even death - illustrating the importance of immunity to the survival of the organism.

Host organisms have two main tiers of defence against foreign infections: innate and adaptive immunity[2]. *Innate* immunity describes the initial defence an organism mounts against infection. A healthy organism develops with this system fully functional. *Adaptive* immunity matures over time and mediates a more effective response to any subsequent infection. There are two types of adaptive immunity: *humoral* and *cell-mediated immunity*. Each consists of a separate set of cells and molecules that protect against extracellular and intracellular microbes, respectively.

1.1.1 CELLS OF AN IMMUNE RESPONSE

Skin, mucus in the natural openings of the body, epithelial barriers, and the specialised cells that reside in these barriers provide a first line of defence against the entry of microbes in the innate immune system[1]. The specialized cells include phagocytes (also called macrophages) which engulf and breakdown *antigens*. Antigens are substances produced by microbes, and, in addition, include virus, bacteria, fungus, parasites, carcinogens, pollution, and a vast number of toxins. When an antigen enters the body it normally elicits an *infection*. The classical signs of an infection are often acute inflammation characterized by *activation* of a number of immune cells, including macrophages and dendritic cells (DC). Activation of these cells stimulates release of

inflammatory mediators that account for the clinical signs of infection. These inflammatory mediators increase blood perfusion to the infected area (inflammation) that allow for the rapid recruitment of a group of specialised cells, collectively called *leukocytes.*

Extravasation describes the movement of the various leukocytes critical to the initiation and eradication of an infection and involve chemokine and receptor-mediated migration and adhesion[2]. The products of the cells involved in the infection also promote cell surface receptor expression on immune cells in the blood stream enabling them to roll slowly along the endothelial surface in order to migrate and stop at the infection site. The complement, coagulation, and fibrinolysis cascade systems also act in unison to initiate, propagate and maintain this inflammatory response.

1.1.1.1 Leukocytes

Leukocytes are a diverse group of specialised immune cells that protect the body against infection and foreign matter[1]. There are five in total, distinct in function and often characterised as *granulocytes* or *mononuclear cells*. Granules of membrane-bound enzymes in the cytoplasm that digest endocytosed particles characterise granulocytes, also called polymorphonuclear leukocytes[3]. There are three types of granulocytes*:* neutrophils, basophils, and eosinophils*.* Mononuclear leukocytes are characterised by the absence of granules in their cytoplasm. There are three types: monocytes, macrophages and *lymphocytes (see Fig 1)*.

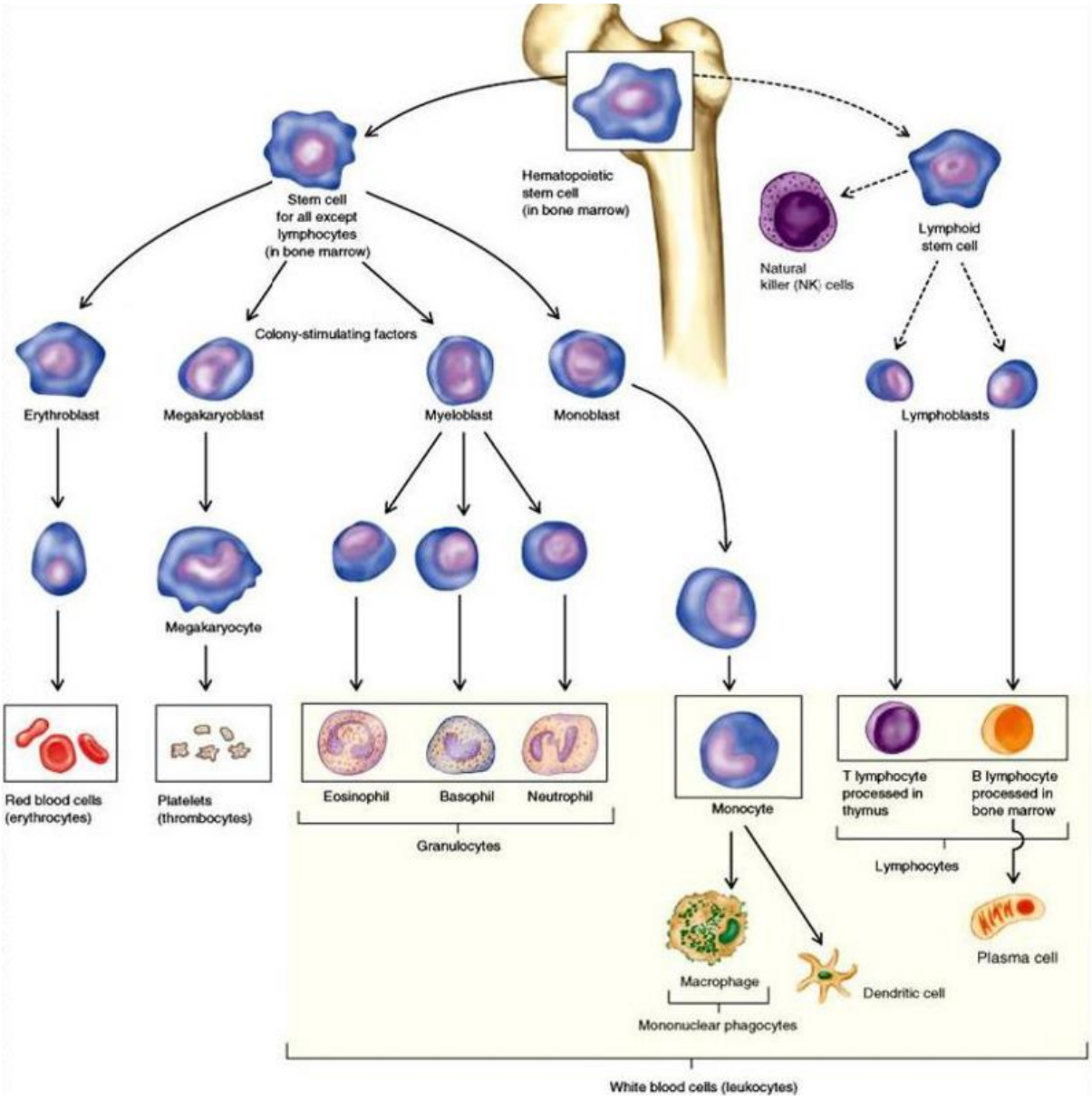

Figure 1. Differentiation of human leukocytes. *A hematopoietic stem cell can differentiate into two lineages: (to the left)into stem cells that differentiate into precursors that eventually become either red blood cells, platelets, the two mononuclear cells, Monocytes and Macrophages, or the granulocytes (Eosinophils, Basophils and Neutrophils); or (to the right) into lymphoid stem cells that differentiate into either Natural Killer (NK) cells or lymphoblasts that become either T or B lymphocytes[3].*

1.1.1.2 Lymphocytes

There are three classes of mammalian lymphocytes: T-cells, B-cells and Natural Killer cells (NK cells). The populations of these lymphocytes are kept relatively stable throughout an organism's lifespan[4]. This homeostasis is achieved through a tightly regulated system of targeted apoptosis

and autophagy involving antigens, cytokines (small, hormone-like extracellular messenger molecules) and other co-stimulatory signals. Naive lymphocytes can live a surprisingly long life and spend most of their existence circulating through the lymphatic system. T cell lineage is differentiated by the type of protein expressed on the surface of the cell[3]. "CD" denotes "Cluster of Differentiation" and is the standard nomenclature used to differentiate T cell phenotypes.

Natural Killer cells (NK cells) are a part of the adaptive immune response[1]. They are a type of cytotoxic lymphocyte that provides rapid response to virally infected cells and tumour formation. They are active around three days after infection.

B cells have three main tasks: generate antibodies against antigens, function as *antigen-presenting cells (APC; see below)*, and develop into memory B cells after activation. The B cell is a key component in *humoral immunity* ("humours" = extracellular fluids of the body), which includes secreted antibodies, complement proteins and certain antimicrobial peptides. B cells can be distinguished from other lymphocytes by several cell surface markers. One such marker, the protein complex known as a *B cell receptor (BCR),* allows the B cell to bind to specific antigens.

T cells also express antigen receptors on their surface. This receptor is called a T cell antigen receptor (TCR). That BCRs and TCRs are formed randomly in order to recognize any antigen make B and T cells key mediators of *adaptive immunity*.

1.1.2 T CELLS

1.1.2.1 T cell subtypes

There are five main T-cell subtypes, all expressing either the CD4 or the CD8 surface marker[5]. The CD4 and CD8 molecules serve to stabilize the interaction between the TCR complex and the Major Histocompatibility Complex (MHC II and I) protein molecules, expressed on various other cells, including APCs. The subgroups of T cells include: "*Regulatory" T cells* (Tregs)which

suppress immunity; whereas "*Natural Killer" T cells* (not to be confused with NK cells) act in a similar way to cytotoxic T cells but are part of the innate response and do not adapt in response to an antigen or infection. The largest groups of T cells are made up of the *"Helper" T-cells* or CD4+ T cells, and the "*Cytotoxic" T-cells* or CD8+ T cells which are the basis for the development of "*Memory" T cells. Memory T cells* are hallmarks of a highly developed organism and will expand more rapidly into helper or cytotoxic T-cells in response to re-infection. CD8+ T cells (cytotoxic T cells, TC cells, or CTLs) recognise their targets by binding to antigens associated with MHC I – present on all nucleated cells. They destroy virally infected cells and tumour cells and are implicated in transplant rejection[6]. CD8+ cells can be inactivated by certain molecules such as IL-10 and adenosine secreted by Treg cells. This forces the CD8+ cells into an anergic state with prevents uncontrolled proliferation of immune cells and helps prevent autoimmune diseases.

1.1.2.2 CD4+ T cells

"Helper" T cells or CD4+ T cells are essential in the co-ordination and amplification of an effective immune response to foreign contaminants as they regulate both humoral and cellular immunity [1]. Cytokines excreted from the cells activate macrophages; promote inflammation; and stimulate the proliferation and differentiation of other T and B lymphocytes *(Figure 2).*

Differentiated effector CD4+ cells appear 3-4 days after infection, the best known being *Th1* and *Th2* - differentiated only in the type of cytokine they produce[7]. Th1 cells secrete *Interferon-y (IFNγ)*, a potent stimulator of macrophages and the production of antibody isotopes that promote phagocytosis of microbes. Th2 CD4+ leukocytes secrete interleukin (*IL)-4*, a cytokine that stimulates the production IgE antibodies from B cells, and *IL-5* which activates eosinophils – therefore promoting phagocyte-independent, eosinophil-mediated immunity useful for targeting parasites. A fraction become long-lived memory T cells.

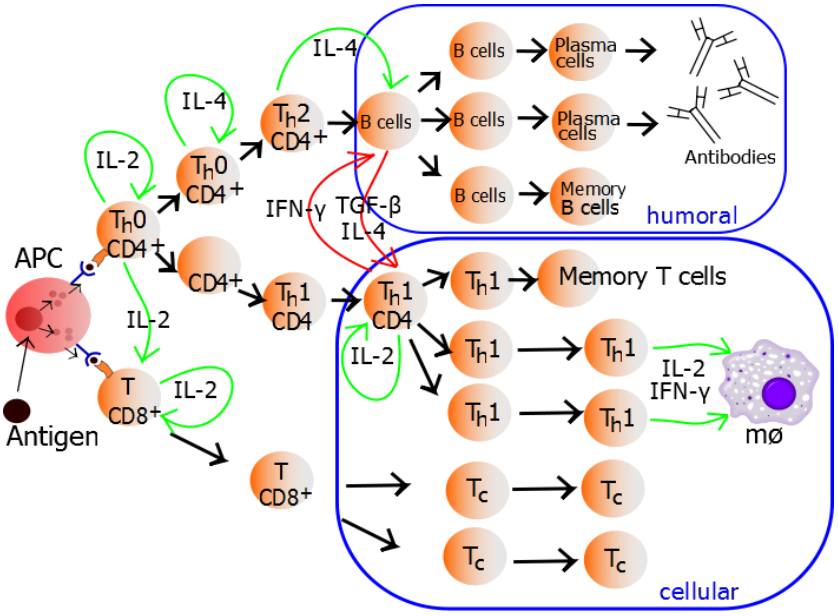

Figure 2. The co-ordination of cell-mediated and humoral immunity. *T Helper cells co-ordinate immunity through release of cytokines that stimulate B cells and cytotoxic T cells. Cytokines also function in an autocrine fashion to reinforce activation[8].*

The importance of the CD4+ T cell phenotype can be illustrated by the deleterious affects the HIV virus has on the immune system: the HIV virus targets cells that express the CD4 surface protein. The subsequent decrease in CD4+ T cell population increases the number of pathogens that escape antigen recognition. As a consequence there is an inflation in the number and severity of infectious diseases in the individual [9].

1.1.3 T CELL ANTIGEN ACTIVATION

T cells are unable to recognize free pathogens in the circulation. A pathogen must first be processed and "presented" to a T cell by an antigen-presenting cell (APC)[1]. T cell antigen activation requires the complexing of a T cell and an APC via the interaction of a series of proteins on the surface of both cells that include either the MHC type I (MHCI) or type II (MHCII) molecules on the APC; the TCR complex; and either the CD4 or CD8 molecule on the T cell. The APC displays antigen molecules on its surface via the MHC molecules to which a CD8+ or CD4+ T cell are able to recognize and initiate a response. An endogenous antigen is displayed by MHC I and an exogenous antigen is displayed on MHCII.

1.1.3.1 Antigen-Presenting Cells

APCs are highly efficient at internalizing antigens, either by phagocytosis or by receptor-mediated endocytosis[10]. APCs capture microbial cells (typically viruses or bacteria) then travel from the infection site to the lymph nodes where the internalized microbe is digested and antigen fragments called *epitopes* are displayed on the MHC molecule on the surface of the cell for T cell activation and clonal expansion *(Figure 3)* [1]. Cells that express both MHC proteins are often termed "Profession APCs"[10]. For example, DCs are one of the most important professional APCs and express a wide range of antigen presentation[11]. A DC, or any cell in the body, that becomes infected by a virus may promote its own self-destruction via a cytotoxic T cell by expressing part of the infected cell through the ubiquitously expressed MHCI complex. However, DC can also promote an immune response via the MHCII proteins and interaction with CD4+ helper T cells. Other types of professional APCs include macrophages, B cells, and some epithelial cells.

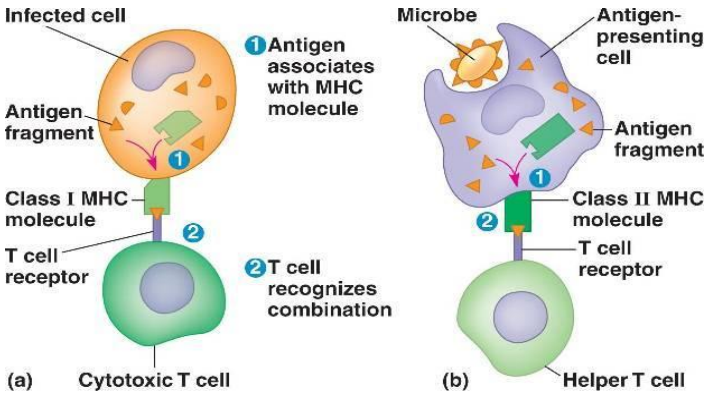

Figure 3. Antigen presentation via Major Histocompatibility Complex I & II and recognition by T Cell Receptors. *1. Antigen associates with MHC molecule; 2. T cell recognizes MHC/Antigen combination*[12].

1.1.3.2 T cell antigen receptors

The T cell antigen receptor (TCR) is a heterodimer that exists bound to the cell membrane in complex with another protein complex, the CD3 molecules. The TCR consists of two variable (α)

alpha and beta (β) chains; the CD3 complex has four distinct chains (a CD3 γ, CD3 δ and two CD3ε chains)[13] – together these molecules form the *TCR/CD3 complex.* The alpha and beta chains of the TCR have a constant (C) region and a variable (V) region. The extracellular variable region binds to the antigen/MHC complex while the constant region anchors the TCR to the cell membrane. As mentioned, during antigen encounter the CD4 and the CD8 molecules work as co-receptors stabilising the interaction between the TCR and the MHCII or MHC I surface molecules. A random process of gene splicing and rearrangement of the segments of DNA that code for the antigen-binding parts of the TCR accounts for the infinite range in antigen specificity [2]. A fully developed population of T cells contains in excess of approximately 1 trillion unique T-cell receptors, which are rearranged and selected for in the thymus. This almost infinite specificity means that upon antigen encounter only one T lymphocyte will be activated and start to proliferate. This process is called *clonal expansion.*

1.1.3.3 Some molecules involved in T cell activation

T cell activation requires two separate signals[1]. The first is the binding and recognition by the TCR to antigens presented by an APC via the MHC. The second signal occurs upon co-stimulation of the CD28 molecule which is expressed constitutively by naïve T cells. The ligand for CD28 is the CD80 protein on the APC. This second, co-stimulatory signal ensures that the cell reacts to foreign contaminants only and strengthens the bond between the two cells[1]. Another interaction between the adhesion molecules integrin protein LFA-1 on the T cell and ICAM on the APC, further strengthen the bond during T cell activation[13]. TCR-antigen recognition activates SRC kinase Lck which phosphorylates the immunoglobulin family tyrosine (Y)-based activation motifs (ITAMs) in the CD3 protein complex[14]*(figure 4).* The subsequent phosphorylation of chain associated protein (ZAP70) leads to the phosphorylation of other protein tyrosine kinases (PTKs), increased endogenous Ca^{2+} flux and RAS activation. These upstream events lead to a complex series of signaling cascades crucial for a normal immune

response that lead to the activation of transcription factors, production of the late phase T cell specific growth factor IL-2 and subsequent clonal expansion[15].

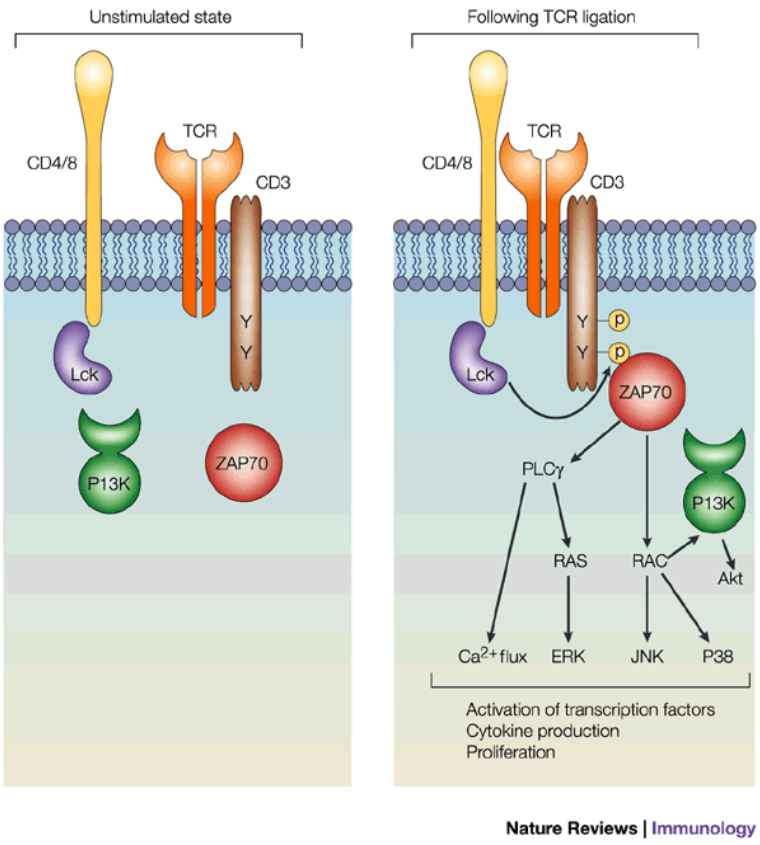

Figure 4. Events following TCR activation. *The T-cell receptor (TCR) and CD4 or CD8 co-receptors recognise the major histocompatibility complex (MHC) (not-pictured). Ligation activates the SRC kinase Lck (purple) which phosphorylates immunoglobulin family tyrosine Y-based activation motifs (ITAMs) in the CD3 protein complex (Brown – Y-p). Subsequent recruitment and phosphorylation of chain-associated protein (ZAP70) leads to a series of complex signalling cascades leading to, PI3K/Akt activation (green) and increased calcium (Ca^{2+}) flux, activation of transcription factors, cytokine production and proliferation[16].*

1.2 CELLULAR METABOLISM

Indeed, what happens internally after TCR/CD3 activation is a classic example of a cell adapting to its environment by the rewiring its metabolic pathways[17]. This metabolic shift from one state to another allows the cells to grow and replicate and, ultimately, serve their function to eliminate foreign pathogens. It also presents a challenge: how to effectively take up and metabolize the nutrients necessary for optimal cellular growth and replication.

Metabolism defines the complex network of chemical reactions that allows organisms to convert substances into metabolic energy, and biomolecules necessary for daily maintenance in addition to growth and proliferation [18].

The conversion of one substance into another is achieved by a series of reactions commonly called metabolic pathways [19]. For example, proteins ingested by an organism are commonly broken down into the amino acids that fuel *in vivo* protein synthesis. However, amino acids can also be converted into numerous nitrogen-containing substances including nucleotides[20]. In addition, amino acids are also converted into molecules containing sulphur groups such as Acetyl Coenzyme A (AcCoa): a molecule vital to energy metabolisms and to the antioxidant glutathione (GSH) that protects cells from damage. Each step in these series of reactions requires an enzyme which allows the metabolic reactions to occur in a regulated fashion.

This regulatory ability is of most importance for the production of energy. Energy is supplied by adenosine triphosphate ATP - a high energy molecule that fuels the reactions that allow muscles to contract, in addition to the numerous biosynthetic reactions that fuel cellular growth and replication[18]. Glucose derived from dietary and internal stores of carbohydrates (glycogen in animals), amino acids from protein, and fatty acids all contribute to the production of ATP through interrelated metabolic pathways *(Figure 5).*

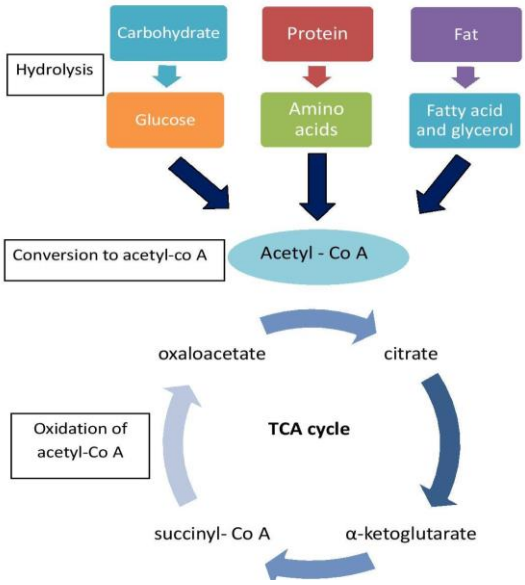

Figure 5. Energy substrates converge on TCA cycle. *The catabolism of the carbohydrate glucose, amino acids from proteins and fatty acids from fat produce Acetyl CoA which can be further oxidized in the Tricarboxylic Acid Cycle (TCA or Krebs cycle) in the mitochondria*[21].

All three metabolic pathways (of glucose, lipids and some amino acids) converge on mitochondrial molecule Acetyl CoA, a key substrate fed into a series of amphibole reactions, also called the *citric acid cycle*, or *tricarboxylic acid* (TCA) cycle[18]. The reactions in the TCA cycle supply the carbon intermediaries needed during the anabolism of numerous biomolecules and the synthesis of ATP. ATP is produced by oxidative phosphorylation by the reducing agent nicotinamide adenine dinucleotide (NADH) and flavide adenine dinucleotide (FADH) at the inner membrane of the mitochondria[22]. Here molecular oxygen (O_2) is reduced to water in the presence of H^+ and electrons from supplied by NADH and FADH. For a simplification see *Figure 6.*

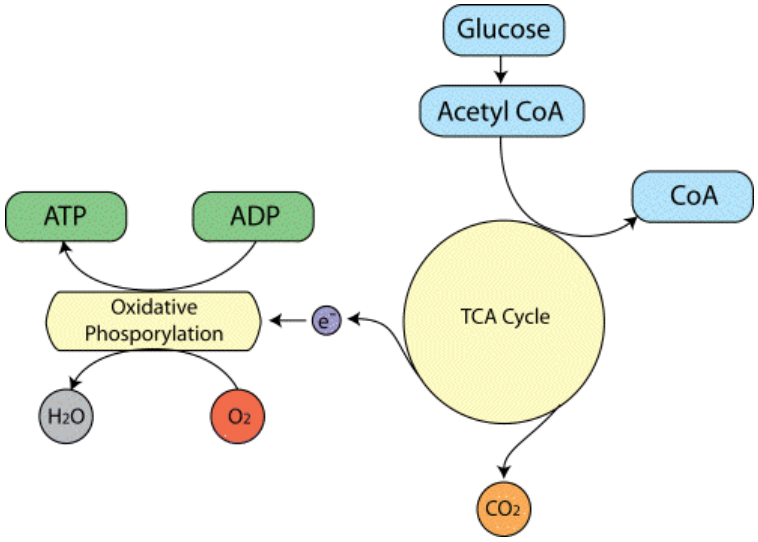

Figure 6. Production of ATP from glucose. *Acetyl CoA is catabolized in the TCA cycle to produce CO_2 and reducing agents for the production of ATP in the mitochondrial matrix membrane[23].*

The energy requirements of cells can change exponentially from moment to moment, depending on the local and systemic requirements[17;24]. To meet such requirements flux through the various metabolic pathways changes rapidly by, for example, regulating the activity of a single, or set of, enzymes in a chosen pathway. This type of dynamic switch in metabolic activity is well illustrated when a naïve quiescent T cell is activated, resulting in growth and proliferation.

1.2.1 T CELL METABOLISM

1.2.1.1 Naive T cells

Naive T cells roam the circulation in search of pathogens[17]. As such their metabolic needs are relatively modest, needing only sufficient ATP to fuel migration and basic housekeeping duties such as maintaining membrane ion gradients and the cytoskeleton, synthesis of biomaterials for cell maintenance, and transportation of proteins and other substrates around the cell. They achieve this mainly through the highly efficient catabolism of glucose and other intermediaries through the TCA cycle and mitochondrial oxidative phosphorylation *(as described above)*[25]. Naive T cells are not autonomous but are dependent on extrinsic signals in order to maintain

this basic metabolic profile [26]. A lack in these signals renders the cells unable to adequately absorb glucose and the cells atrophy and die [27].

1.2.1.2 Proliferating T cells

Within minutes after activation, T cells undergo dramatic physiological changes. This can be observed by cell swelling (blastogenesis) during the first 24 to 72 hours post activation[28]. This increase in size is supported by elevated uptake of several energy substrates necessary for the increased synthesis of biomass consisting of DNA, proteins and lipids[25;29-32]. Synthesis of biomass also requires ATP and the electron donor NADPH. Figure 7 *(below)* shows the massive shift in metabolic activity associated with the production of protein, lipids and ATP in activated lymphocytes.

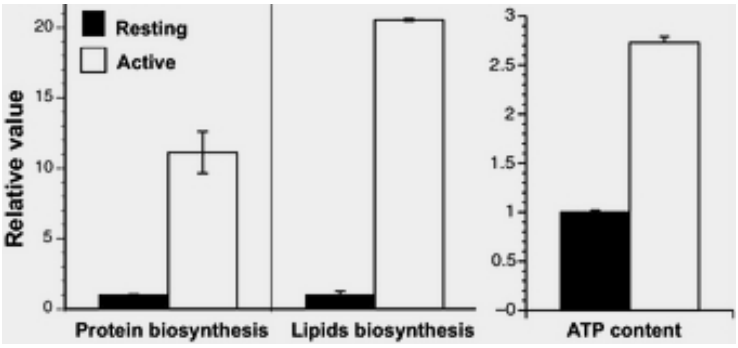

Figure 7. *Increased biosynthesis of proteins, lipids and energy production upon T-cell activation[33].*

The release of cytokines and the up-regulation of several transcription pathways such as the janus-kinase and Phoshatidylinositide 3-kinases / serine-threonine-specific protein kinase (PI3K/Akt) pathways, mediated by the *c-Myc* proto-oncogene further enhance and prime the cell for expansion[17].

Activated T cells produce the T cell-specific growth factor IL-2 which further stimulate adjacent T cells to up-regulate their IL-2 receptor (IL2R) as well as the glucose transporter GLUT1 for increased uptake of glucose, both in order to support proliferation[34]. Downstream from the IL-

2R, the PI3K-Akt transcription pathway potentiates the up-regulation and translocation of Glut1 to the cell membrane; increases the up regulation and localisation of hexokinases and other glycolytic enzymes such as phosphofructo kinase 1 (PFK1) and lactate dehydrogenase (LDH); increases flux through the pentose phosphate pathway (PPP); and activates the mammalian target of rapamycin (mTORC1) pathway vital for protein synthesis – thus ensuring maximum uptake, retention and utilization of glucose within the cell and initiation of protein synthesis *(Figure 8)*[35-37].

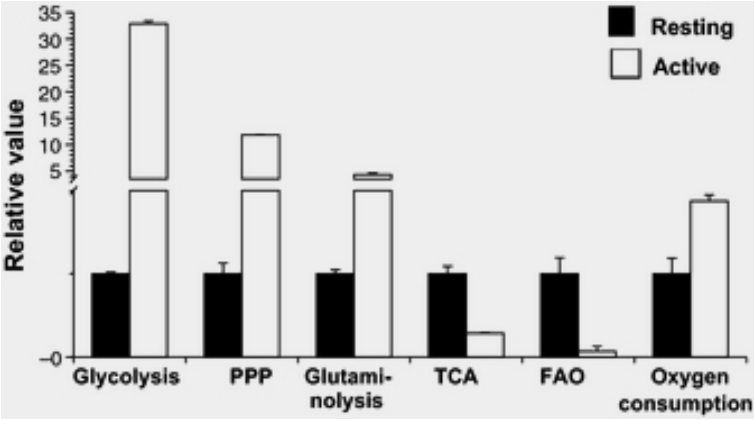

Figure 8. Metabolic reprogramming in activated T cells. *The indicated metabolic activities in resting T cells (black bars) and activated T cells (white bars). The shift was determined 24 hours post activation by increased glycolysis, pentose phosphate pathway activity, glutaminolysis and oxygen consumption, with concurrent reduced activity in the TCA cycle and fatty acid oxidation*[33].

AMP-activated Protein Kinase (AMPK) is a vital regulator of energy metabolism in cells[18;38]. It functions by increasing the activity of catabolic, ATP-generating pathways and inhibiting anabolic processes that deplete ATP. AMPK traditionally responds to an increasing intracellular AMP / ATP ratio which indicates falling cellular energy levels and by phosphorylation of Liver kinase B1 (LKB1) at Threonine 172. However AMPK has been shown in expanding T lymphocytes to respond to elevated Ca^2 levels through Calcium-calmodulin-dependant protein kinase kinases (CaMKKs)*(Figure 9)*[39]. TCR activation evokes the release of Ca^2 from the endoplasmic reticulum in addition to Ca^2 entry via membrane ion channels. A sustained, elevated intracellular Ca^2 concentration is vital during the early stages of T cell activation,

especially for the production of cytokines. In non-proliferating cells AMPK functions to promote ATP production and conservation by promoting ATP-producing processes and down-regulating catabolic processes. However, in expanding lymphocytes AMPK responds to this initial calcium stimulus and functions to "prime" T lymphocytes for the demands of the energy-intensive processes during activation and expansion by accelerating ATP production in anticipation of increased demand.

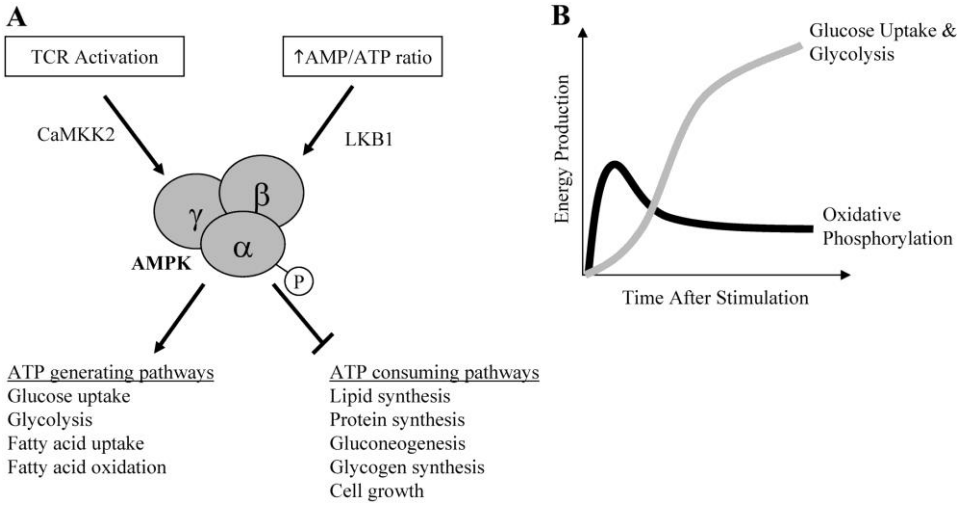

Figure 9. Activated T cells prepare for increased energy demand by activation of AMPK and increased uptake and consumption of glucose. *(A) AMPK activation by TC-induced R activation of CaMKK2. (B) Immediately after TCR activation AMPK up-regulation contributes to increased ATP production through oxidative phosphorylation, followed by increased synthesis and translocation of Glut1 to the membrane for increased glucose uptake and glycolysis[40].*

1.2.2 GLUCOSE METABOLISM IN T CELLS

In resting mature lymphocytes glucose utilisation is divided almost evenly between lactate production (glycolysis), synthesis of oligosaccharides (glycosylation), and oxidation to carbon dioxide (glycolysis, TCA cycle and mitochondrial oxidative phosphorylation)[32]. Activated T cells, however oxidize the majority of the glucose to pyruvate through glycolysis and then to lactate by lactate dehydrogenase (LDH) where it is then excreted from the cell *(see figure 9)*[32;34;37;40-42]. This shift to aerobic glycolysis, also known as the Warburg effect (named after its discoverer), is

elevated and sustained during expansion and differentiation and occurs even in the presence of ample amounts of oxygen[43].

At first glance this shift to a less efficient production of ATP seems counterintuitive to the energy demands of proliferating cells: the oxidation of glucose through aerobic glycolysis produces only 2 molecules of ATP whereas 36 ATPs are generated through mitochondrial oxidative phosphorylation[18]. Yet many single-celled organisms use glucose fermentation to proliferate, demonstrating that aerobic glycolysis is sufficient in providing the necessary ATP for cellular replication. This perceived inconsistency in energy production may be due to our over-estimation of a cell's energy requirements under proliferation. Normal proliferating cells are exposed to an abundant supply of glucose and nutrients and despite tremendous rates of replication consistently exhibit high ratios of NADH/NAD and ATP/ADP, suggesting that the demands of the proliferating cell extend far beyond mere energy production[24]. A proliferating cell must replicate all of its contents. This replication places large demands on the supply of macromolecule precursors (lipids, amino acids, nucleotides and reducing agents (such as NADPH). Glucose (and glutamine, discussed in the next section) provides, almost exclusively, the carbon backbones necessary for this synthesis through anabolic pathways: the PPP and the production of the sugar metabolite ribose 5-phophate necessary for nucleotide synthesis (not pictured), and NADPH which is also necessary for the maintenance of reduced GSH in addition to macromolecule synthesis; fatty acid synthesis from Acetyl CoA; and glycolytic intermediaries for synthesis of non-essential amino acids (NEAA) *(see figure 10)*[28;44]. It quickly becomes apparent that committing the majority of glucose to oxidative phosphorylation and mitochondrial ATP synthesis would severely restrict production of these intermediaries and retard cellular replication[28].

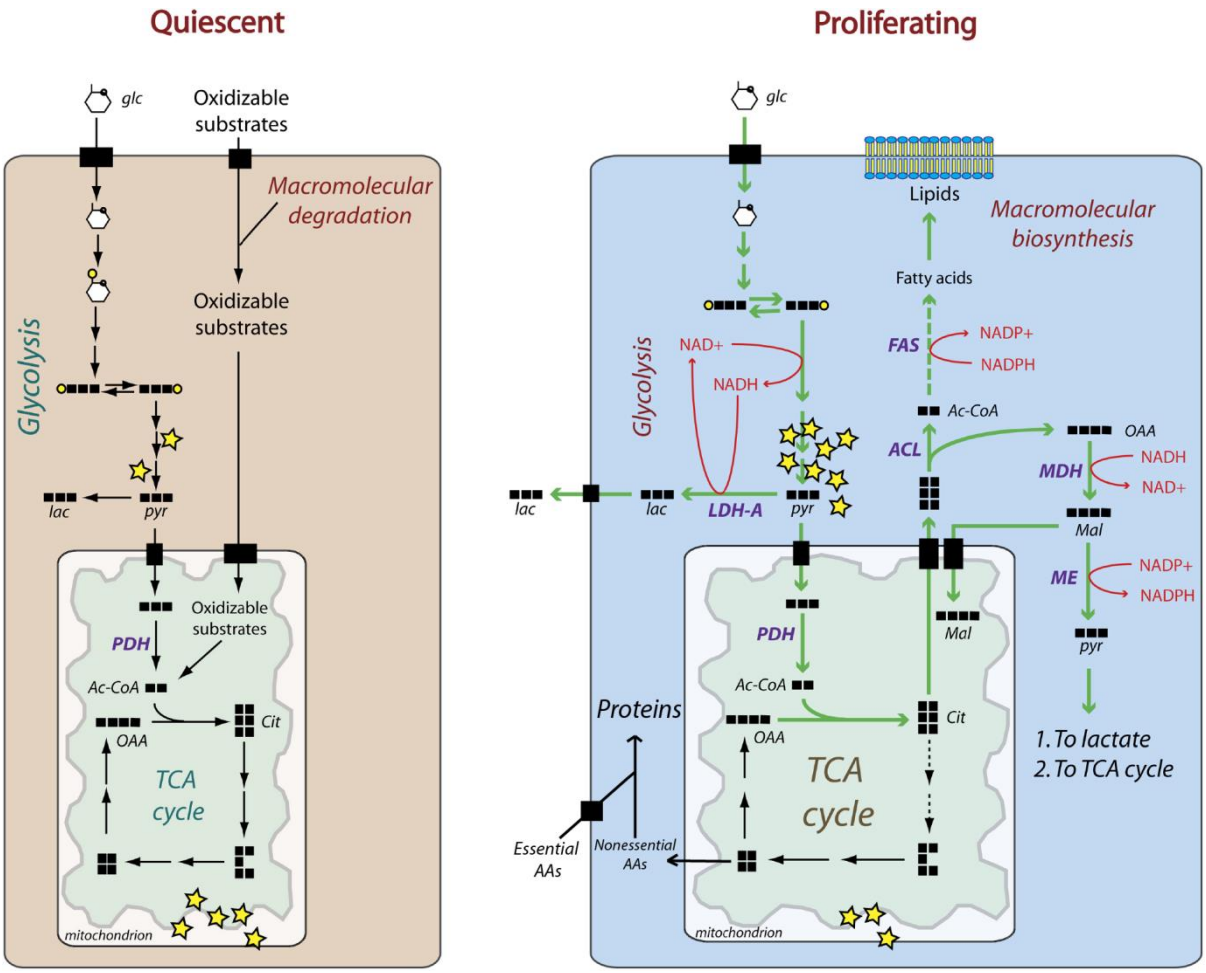

Figure 10. Flux of glucose and substrates in quiescent and proliferating cells. *Flux of glucose (glc) in a quiescent cell (left) through glycolysis into pyruvate (pyr) which is then oxidised in the TCA cycle. Other oxidisable substrates like amino and fatty acids can also be converted into TCA intermediaries. Here the majority of ATP (yellow stars) is produced by oxidative phosphorylation. During proliferation (right), ATP generation is due largely to the massive increase in glycolytic flux. The resulting pyruvate is converted into lactate (lac) by lactate dehydrogenase (LDH) which regenerates NAD+ from NADH necessary for glycolysis. The resulting lactate is excreted from the cell. Some pyruvate enters the mitochondria to be converted to acetyl-CoA by pyruvate dehydrogenase (PDH) which is then converted into other TCA intermediaries such as citrate (cit), essential in the generation of lipid membranes for daughter cells. Amino acid pre-cursors from the TCA cycle also contribute to the synthesis of proteins in the cell. In the cytoplasm citrate is hydrolysed by ATP citrate lyase (ACL) into acetyl-CoA for synthesis of lipids by fatty acid synthase (FAS). Malate dehydrogenase (MDH) converts the resultant oxaloacetate into malate (mal) which can then either by transported back to the mitochondria for conversion back to pyruvate by malic enzyme (ME) whilst generating NADPH used in the synthesis of fatty acids[24].*

Of perhaps equal importance as a major provider of carbon backbones for this increase in biomass by proliferating T cells is glutamine.

1.2.3 GLUTAMINE METABOLISM

Glutamine is the most abundant amino acid in the human body comprising 60% of free amino acids[45]. It is synthesised primarily in the lungs, skeletal muscle and adipose tissue. Serum concentrations range from 0.4 - 0.9mM[46]. It is synthesized through the conversion of glutamate and ammonia to glutamine through the hydrolysis of ATP:

L-Glutamate + NH_3 + ATP > L-Glutamine + ADP + Pi

Glutamine is the primary supplier of nitrogen and carbon for protein synthesis; it is essential in the transportation and regulation of nitrogen; vital in the production of nucleotides, glucose through gluconeogenesis, and glucosamine; as a precursor in the synthesis of neurotransmitters; acid/base regulation in the kidneys; and in the replenishing of GSH for optimal protection against oxidative stress[47;48]. It is also the major provider of carbon for alpha-ketoglutarate and an important anaplerotic substrate for the TCA cycle and the synthesis of lipids *(Figure 11).* Indeed, many highly proliferative tissue types exhibit elevated levels of glutamine turnover to support energy production including enterocytes, fetal cells, hair follicles, tumours and the cells of the immune system[46].

Activated T cells selectively enhance glutamine uptake through expression and synthesis of the major glutamine transporters Sodium-coupled amino acid transporters 1 and 2 (SNAT1 and SNAT2), Solute carrier family 7 member 5 (SLC7A5) and Neutral amino acid transporter (ASCT2). Elevated glutamine uptake and metabolism is stimulated by TCR-CD3 induced ERK/MAPK activity and increased *c-myc* expression [30;30;49].

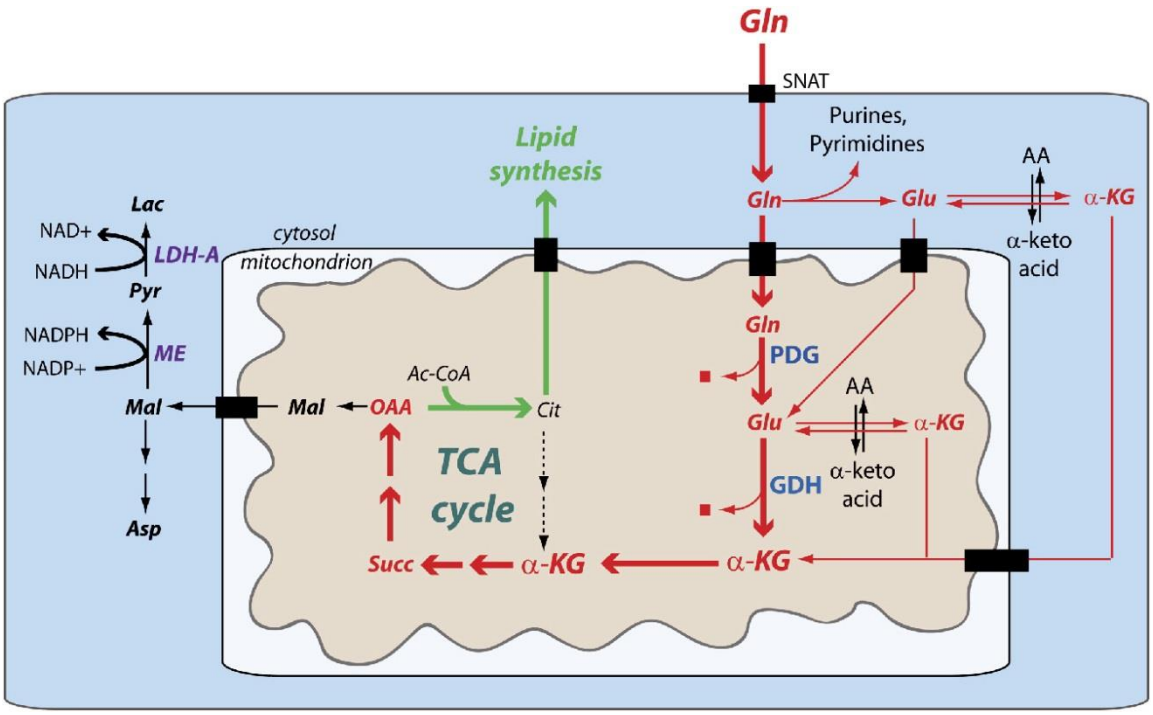

Figure 11*. *Catabolism of glutamine in proliferating cells. *Citrate lost to the synthesis of lipids (green arrows) in proliferating cells is replenished from the metabolism of glutamine (red arrows). After transport into the cytosol by SNAT glutamine (Gln) donates nitrogen for purine and pyrimidine synthesis through oxidation to glutamate (Glu) by Glutaminase (GLS). Glutamate donates its amino group to α-keto acids for synthesis of nonessential amino acids and α-ketoglutarate (α-KG). Mitochondrial glutamine can also be converted into glutamate by phosphate-dependent Glutaminase (PDG) which frees an amido group (red square). The mitochondrial glutamate can then either be used to synthesize amino acids by aminotransferases or mitochondrial α-KG by glutamate dehydrogenase (GDH). α-KG can then enter the TCA cycle and replenish oxaloacetate (OAA) and citrate. The carbon from glutamine can also be converted into lactate (glutaminolysis) which generates the reducing agents NADPH and NAD+ in the cytoplasm[24].*

As figure 11 demonstrates, glutamine is a key anaplerotic source of carbon for replenishment of TCA intermediaries and synthesis of macromolecules. It is also a source of fresh NAD+ and NADPH necessary for reductive, biosynthetic pathways. In glioblastoma cells 90% of glucose and 60% of glutamine is converted into either lactate or lanaline which is then rapidly excreted from the cell, with robust NADPH production being the result[50]. Inhibition of LDH, a key enzyme in this process, undermines cell proliferation. This suggests that the removal of excess carbon from the cell may be necessary to generate enough NADPH to fuel cell proliferation[51].

As mentioned earlier, GSH is the cell's major antioxidant and its synthesis is dependent on adequate supply of glutamine. GSH's role is crucial in preventing oxidative damage to DNA,

proteins, enzymes and lipids[52]. It is also an essential component in the synthesis and repair of DNA, enzyme activation and protein synthesis – all critical under rapid cell proliferation. GSH is synthesised from the amino acids cysteine, glycine and glutamate. Due to this dependence on glutamate, GSH synthesis is also highly dependent on adequate cellular glutamine. Restricting available GSH to proliferating lymphocytes greatly diminishes their ability to replicate[53].

1.2.3.1 Glutaminase

Glutaminase catalyses the hydrolysis of glutamine to glutamate and ammonia:

$$\text{L-glutamine} + H_20 > \text{L-glutamate} + NH_4$$

Three mammalian Glutaminase isoforms have been identified: the Kidney-type Glutaminase (KGA) and Glutaminase C (GAC) - a splice-variant of KGA - both coded by the *gls* gene; and Liver-type Glutaminase (LGA) - coded by *gls2*. KGA and GAC are expressed in the brain, kidney, intestine, fetal liver and many other tissues, unlike LGA, which, as the name suggests, is located only in hepatic cells[46]. Evidence points to the binding of both KGA and GAC to the inner membrane of the mitochondria, however some evidence suggests they may reside in the intramembrane, free from the membrane[54;55]. Figure 12 depicts the identical N-terminus shared by KGA and GAC and the identical core shared by all variants that is highly conserved in each isoform and likely the catalytic domain of the enzyme. Each enzyme has a unique C-terminal.

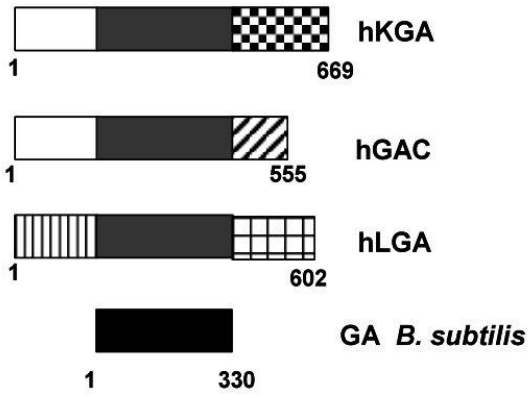

***Figure 12. The three human glutamines isoforms are compared to bacterial Glutaminase**. A common core (black) is conserved in each isoenzyme. GAC and KGA isoforms share an identical N-terminal (white). Each human Glutaminase isoform has a unique C-terminal (patterns)[56].*

The three different isoenzymes vary not only in protein structure but also in enzyme kinetics. GAC and KGA isoforms require a polyvalent anion for activation, the most common being phosphate which elicits half-maximal activity at 20-30mM[46]. The isoforms have K_m values for glutamine at around 2-5mM. The Km values for both enzymes are improved in the presence of elevated phosphate[30]. This is in contrast to LGA which has a relatively high K_m for glutamine (17 mM) and hence much less sensitive to variations in the concentration of phosphate. Both KGA and GAC, but not LGA, are inhibited by their enzymatic product glutamate.

Reinforcing the notion that fast-growing cells are reliant on increased levels of glutamine, many cancer cells up-regulate the KGA and GAC isoforms for continued expansion, with mRNA levels elevated in breast cancer, colorectal carcinomas, adenomas and glyomas[29].

1.2.3.2 Glutaminase inhibitors

Recently, attention has been focused on the ability of two compounds found to inhibit mammalian Glutaminase activity *in vitro* and in tumour cells[57-59].

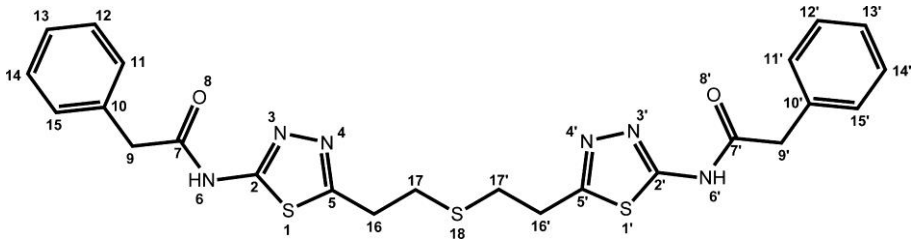

Figure 13. *BPTES (Bis-2-(5-phenylacetimido-1,2,4-thiadiazol-2-yl) ethyl sulphide)*

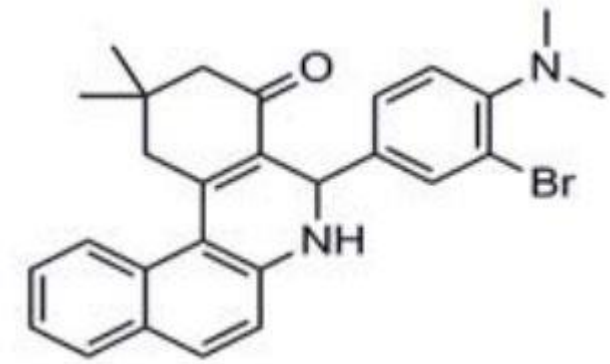

Figure 14*. Compound "968" (5-(3-bromo-4-(dimethylamino) phenyl)-2,2-dimethyl-2,3,5,6-tetrahydrobenzo[a]phenanthridin-4(1H)-one)*

Both compound Bis-2-(5-phenylacetimido-1,2,4-thiadiazol-2-yl) ethyl sulphide (BPTES) *(figure 13)* and 5-(3-bromo-4-(dimethylamino) phenyl)-2,2-dimethyl-2,3,5,6-tetrahydrobenzo[a]phenanthridin-4(1H)-one (968) *(figure 14)* appear to allosterically regulate the Glutaminase enzyme by altering the tetramer confirmation[59]. This differs to other Glutaminase inhibitors such as 6-Diazo-5-oxo-L-norleucine (DON) and acivicin which covalently modify residues on the binding pocket and have significant toxicity issues. Both inhibitors are neither irreversible nor competitive versus glutamine and simultaneously lower both K_m and V_{max} values[58]. It appears that 968 has a reduced ability to inhibit the phosphate-activated Glutaminase, whereas BPTES can, hinting at differing modes of inhibition for each compound.

Both KGA and GAC Glutaminase isoforms are elevated in certain tumour cells[60]. Recent research has shown that inhibition of the GAC enzyme by compound 968 in these tumours can inhibit oncogenic progression[59].

1.3 AIM OF THE STUDY

Based on what has been described above we hypothesize that the proliferative phenotype of increased glucose and glutamine dependency seen in many different cancer cells is similar in activated mammalian T lymphocytes , a view which is supported by the literature[28;29;41;50;61]. The enzyme Glutaminase is of particular interest because of it essential role in the catabolism of glutamine for anaplerotic carbon and reducing agents vital to cell proliferation.

The primary aims for the study, therefore, are as follows:

- Identification of protein and mRNA expression for Glutaminase in naïve non-proliferating and CD3/CD28-activated proliferating CD4+ T lymphocytes.
- Investigate the requirement of glutamine for T cell proliferation by selective inhibition of Glutaminase and by glutamine removal.
- Investigate the intracellular concentrations, and flux of glutamine, in proliferating CD4+ T lymphocytes

2.0 METHODS

A complete list of solutions and reagents can be found in *Appendix B.*

2.1 CD4+ T Cell Isolation

Human CD4+ T lymphocytes were isolated from buffy coats (supplied from Blodbanken, Ullevål sykehus). Buffy coats are samples of anti-coagulated blood rich in leukocytes and platelets after density gradient centrifugation of whole blood samples. Average volume was approximately 40-50ml. Buffy coats were diluted with 24ml cell medium (RPMI 1640: 10% inactivated FBS (fetal bovine serum); 2mM L-glutamine; 0.5% Penicillin-Streptomycin) and 1ml EDTA (Ethylenediaminetetraacetic acid) and rotated for 15min at 4 °C to prevent coagulation.

Dynabeads CD4+ Isolation

The Dynabeads CD4 Positive Isolation Kits (Invitrogen) were used to isolate CD4+ T lymphocytes from buffy coats. The Dynabead vial was first vortexed for 30 seconds, then 200uL of the super paramagnetic bead solution (4.5 μm diameter; 4 x 10^8 beads/mL) was suspended in a Dynamag-2 magnet (Invitrogen), the supernatant removed, and the bead pellet washed with 1ml PBS (phosphate buffer solution) at room temperature to remove the 0.02% sodium azide preservative. The beads were then resuspended in 200uL cell medium and then added to the buffy coat solution which was then rotated at 4 °C for a further 30 minutes to allow bead/cell binding. The bead-bound cells were then removed from the buffy coat solution by dispending the solution into two 50ml Falcon conical tubes and suspending these in a DynaMag-50 magnet (Invitrogen) for 5 minutes to allow the bead-bound cells to migrate and adhere to the side of the tube. The buffy coat supernatant was then gently removed and discarded. The resulting bead/cell deposit was then collected into one 15ml Falcon tube using 5ml PBS, then placed in a

Dynamag-5 (Invitrogen) for 2 minutes. This step was repeated 4 times to wash the bead-bound CD4+ T lymphocytes and remove any impurities. The cell pellet was then suspended in 100uL DETACHaBEAD solution (Invitrogen) and incubated for 1 hour at room temperature with gentle mixing. The DETACHaBEAD solution contains a polyclonal anti-Fab antibody that releases the CD4+ cells from the Dynabeads. After 1 hour the solution was vortexed at 2500rpm/min for 10 seconds and placed in a magnet for 1 minute. The supernatant containing the released CD4+ lymphocytes was then transferred to a fresh 15ml tube. The remaining beads were then washed with 1ml PBS and then placed back on the magnet where the supernatant was removed and added to the fresh tube. This step was repeated twice to salvage any residual cells. The resulting 2-2.5ml suspension was then placed in the magnet for a further 2 minutes to remove any remaining beads then centrifuged for 7 min at 1500rpm after which the supernatant was discarded The cell pellet was then washed in 5ml PBS then centrifuged again for 7 min at 1500rpm. After discarding the supernatant the cell pellet was suspended in 1ml of cell medium *(described earlier)* and the cells were counted using a Biorad TC10 Cell Counter (isolation using this method yielded on average 50 million CD4+ lymphocytes) and incubated in cell medium at a concentration of 5 million cells/ml and stored (if not used immediately) in a cell incubator at 36.5°C; 5% CO_2.

2.2 Identification of Glutaminase isoforms in resting CD4+ cells

Western blots were used to characterise the relative levels of the Glutaminase isoenzymes in resting mature CD4+ lymphocytes.

Lysation of cell pellet

To effectively lyse the CD4+ cell membrane a solution containing 100uL of NP40 lysis buffer (Novex), 1uL protease inhibitor phenylmethanesulfonylfluoride (PMSF; 100x; Sigma) and 1uL of protease inhibitor cocktail (100x; Sigma) were added to the cell pellet and resuspended several times. The solution was left on ice for 15 minutes with occasional vortexing (once every 3

minutes; 2500rpm/min). To release the mitochondrial Glutaminase enzymes this procedure was supplemented with sonication (amplitude 60%; 6 seconds: 2x 2 second bursts). The twice-lysed cell solution was then centrifuged (15000RPM; 1 minute). The supernatant was then removed, and, if not used immediately, snap frozen by suspending the supernatant in a -78°C ethanol/dry ice solution and then stored in a -80°C freezer.

Protein concentration

See *Appendix C* for a complete list of protein concentrations.

Nanodrop 2000c Spectrophotometer

Proteins in solution absorb ultraviolet light at wavelengths peaking at approximately 280nm. The NanoDrop 2000c Spectrophotometer (Thermo Scientific) was initially used for protein concentration measurements. Two uL of the NP40 lysis buffer were pipetted onto the measurement pedestal of the Nanodrop 2000c and a BLANK reading was initiated using the PC software (Group: Classic; Protein A280). The pedestal arm was raised and the sample wiped from the upper and lower pedestals using a lint-free laboratory wipe. Next, 1-2uL of the CD4+ lysate was pipetted onto the lower pedestal, the sample named in the PC program and a measurement initiated. A spectral image was reviewed to assess sample quality – a density peak should form at 280nm wavelengths with sufficient protein. The measurements were repeated for all samples and data exported to excel.

Pierce BCA Protein Assay

Protein measurement was later performed using the Pierce BCA protein assay (Thermo Scientific). This method was used on selected samples as high NP40 buffer has relatively high A280 absorbance along with other non-protein components such as nucleic acids and insoluble cell lysates which also absorb UV light at 280nm. Together this contributed, in some cases, to unreliable and inconsistent measurements using the A280 method in the Nanodrop 2000c

Spectrophotometer (Thermo Scientific). The BCA protein assay is based on bicinchoninic acid (BCA) and relies on two reactions: first, the peptide bonds in proteins reduce Cu^{2+} in proportion to the total amount of protein present in the solution, then the resulting Cu^{+} ions chelate with two molecules of bicinchoninic acid to form a purple-coloured product that strongly absorbs light wavelengths of 562nm. The protein concentration can then be assessed by measuring the absorption characteristics of the target samples against protein solutions with known absorption characteristics. *The procedure for this assay can be viewed in Appendix A.*

Preparation of protein samples for polyacrylamide gel electrophoresis (PAGE)

The Criterion Precast polyacrylamide gels (Biorad) come in various percentage densities of acrylamide. The acrylamide density chosen is dependent on the size of the protein of interest: large proteins should be run in low-percentage acrylamide gels and small proteins should be run in higher percentage gels. A 7.5% density gel was chosen because of the small size of the three Glutaminase isoforms (KGA: 73kDa; LGA: 66kDa; GAC: 55kDa). Each well in the gel should hold the same amount of total protein. This required the dilution of the higher-concentrate samples with the NP40 lysis buffer to standardize the concentrations. Next, the lysate was mixed with 2x sodium dodecyl sulphate (2xSDS) sample buffer at 1:1 ratio *(see Table 1 for a complete list of solutions).* The SDS solution binds to and uniformly charges the proteins in the sample so they can be separated according to relative size (and not charge). The maximum well volume is determined by the number of wells in the gel and was taken into account when calculating lysate and SDS sample buffer volumes. The SDS sample buffer/lysate mixture was heated for 5 minutes at 96 °C to denature the proteins.

Loading samples

A Criterion Precast Gel (Biorad) was placed in a Criterion Cell (Biorad) PAGE container prior to sample loading *(see Table 2 for a complete equipment list).* The container was filled with SDS running buffer, including the reservoir at the top of the gel. Potential bubbles at the bottom of

the gel were removed by lifting the gel a little upwards in the buffer once or twice. The first well was loaded with 10uL Precision Plus Dual Colour (Biorad) protein standard (not heated). The boiled samples were loaded carefully into the other wells with a pipette.

Table 1. *List of solutions*

Solutions	Components
SDS Running Buffer	150 g Tris, 720 g Glycine, 50 g SDS, dH2O up to 5 L
Transfer Buffer	15 g Tris, 72 g Glycine, 1 L (20%) methanol, dH2O up to 5 L
TBS (10x)	12.11 g Tris, 58.4 g NaCl, pH 7.5
TBST	1/10 10x TBS + 9/10 dH2O , 0.1% Tween 20
Blocking Buffer	5% non-fat dry milk powder in 1x TBST
Primary Antibody	Diluted in 1x TBST
Secondary Antibody	8.4 µl from a 40% glycerol antibody solution to 10 mL of 1x TBST

Table 2*. List of equipment*

Equipment	Manufacturer	Catalog #
Criterion 12+2 well 7.5% Precast gels	Biorad	345-0005
Criterion 18 well 7.5% Precast gels	Biorad	345-0006
Criterion Cell	Biorad	135BR
Criterion Blotter	Biorad	560BR
Criterion Filter Paper	Biorad	170-4085
Criterion Foam Pad	Biorad	170-4086

Electrophoresis

The electric current generated by the PAGE cell forces the negatively charged protein samples to migrate through the acrylamide gel from the negative to the positive electrodes, where they fractionate according to size. After loading the wells, the PAGE container was run with a Biorad Powerpac 300 at 100V for approximately 10 minutes until the samples had entered the gel properly, then at 130V until the dye had completely passed through the gel (typically 1 – 1.5 hours).

Protein transfer

Once electrophoresis was completed the proteins were then transferred to, and immobilized on, a polyvinylidene fluoride (PVDF) membrane (Millipore). The membrane is highly non-reactive and has a non-specific affinity for amino acids which makes it resistant to solvents and easily reused when examining other proteins. First, the gel was removed from its plastic housing and

sandwiched in a sandwich holder (Biorad) next to a similar sized piece of PVDF membrane, compressed on either side by three 9.5x15.2cm Criterion Filter Papers (Biorad) and a Criterion Foam Pad (Biorad). Care was taken to ensure gel viability and the eradication of air bubbles that would impede transfer. The sandwich was placed in a Criterion Blotter (Biorad) container which was filled with transfer buffer and run overnight at 4 °C with a Biorad Powerpac 300 at 15V for approximately 12-14 hours.

Blocking

Before probing with the appropriate antibody, the freshly blotted PVDF membrane was blocked to prevent any unspecific antibody binding: 1 hour at room temperature in blocking buffer (5% non-fat dry milk powder in TBST (Tris-Buffered Saline plus Tween 20) on a roller. Milk contains many proteins that bind to the membrane and reduce non-specific binding. After blocking the membrane was rinsed with TBST until the rinsing liquid was clear.

Probing

Primary antibodies used for detection are produced when a host animal is exposed to the protein of interest and the resultant antibodies harvested. When exposed to the membrane-bound protein the antibodies bind to the protein. A secondary antibody is then directed at a species-specific segment of the primary antibody (i.e. mouse, rabbit, donkey). The secondary antibody is linked to horseradish peroxidase which is subsequently used to cleave a chemiluminescent agent that produces light and can be detected by a light-sensitive camera to determine protein concentrations.

The PVDF membrane was first cut into strips to allow incubation with the three Glutaminase antibodies plus the control (α-tubulin) and then incubated in the primary antibody solution (5ml TBST plus antibody) over night at 4°C on a roller. After the primary incubation the membrane(s) was washed with TBST for one hour, changing buffer every 10 minutes.

Incubation with the secondary antibody (8.4uL from a 40% glycerol antibody solution to 10ml of 1xTBST) was 1 hour at room temperature, followed by the same washing procedure as for the primary antibody.

Detection

The probed and washed membrane were incubated with 1-5ml (dependant on number of membranes) of Reagent A (Lumino/Enhancer Solution) and B (Stable Peroxide Solution) from the Super Signal West Pico Chemiluminescent Kit (Thermo Scientific) and laid on a clear plastic sheet (e.g. Gladpack, gladwrap). The membrane was then exposed using the G.BOX Chemi XL CCD system (SynGene) at 2 min intervals for 15 minutes to capture the image.

Re-probing

For re-probing, the membrane(s) were washed with TBST for 30 minutes to remove the chemiluminescent substrate then incubated with the Restore Western Blot Stripping Buffer for 5 to 15 minutes at room temperature, washed in TBST for a further 30 minutes, blocked with blocking buffer *(described previously)* for 1 hour at room temperature and then re-probed.

2.3 CD4+ T lymphocyte activation

CD4+T lymphocyte activation assay

Activation of CD4+ lymphocytes was performed in 24-well incubation plates (approx. 3 mill/ml for activated cells; and 5 mill/ml for control cells). The cells were incubated in RPMI1640 supplemented with glutamine and antibiotics *(as described previously).* CD4+ cells were stimulated with 37.5uL Dynabead CD3/CD28 Expansion beads (Invitrogen; $4x10^7$ cells/mL); the control cells received 22.5uL dummy beads - Dynabead Mouse T-Activator CD3/CD28 (Invitrogen; $4x10^7$ cells/mL). All beads were washed with PBS to remove the preservative as described earlier. IL-2 was added to all wells at a concentration of 30U/mL. Total volume in each well was 1ml. The plate was then incubated at 37*°C* / 5%CO2 for 72 hours in an incubator.

After 72 hours the well contents were then moved to 1.5mL Eppendorf tubes and centrifuged at high speed (15000RPM) for 10 seconds. The supernatant was removed and the cell pellet washed with PBS, centrifuged again at high speed and, if not used immediately, snap frozen in a -78 °C ethanol/dry ice solution and stored in a -80 °C freezer.

[3H]-Thymidine Incorporation

A [3H]-Thymidine incorporation assay was performed to test for successful CD4+ lymphocyte activation. 3H-thymidine is a radioactive nucleoside that is incorporated into the newly synthesised DNA strands of the proliferating cells during mitotic cell division. The protocol above was adjusted to a 96 well plate/100uL volume assay using the same cell-to-bead ratio and activation with CD3/CD28 Dynabeads. After incubation for 48 hours a 25uL solution of [3H]-Thymidine (40 uCi/mL; Nerliens) was added to each well. After incubation for a further 24 hours the cells were harvested using a Filtermate 196 Harvester (Packard) and the level of 3H-thymidine incorporation into DNA then measured using a Top Count Microplate Scintillation Reader (Packard).

7 day expansion assay

Fifty ml of cell medium supplemented with IL-2 (30U/ml) was heated to 36.5°C and added to a 150 cm^2 Falcon Cell Culture Flask (BD Biosciences). Approx. 50 million CD4+ lymphocytes were added to the mixture after isolation *(see CD4+ lymphocyte isolation).* Eight hundred uL of Dynabeads CD3/CD28 Expansion beads ($4x10^7$ beads/ml; Invitrogen) solution was added to the lymphocyte culture (after washing; see *CD4+ T lymphocyte activation assay)* and gently resuspended with a 5ml pipette. After four days the cells were gently resuspended once more and then split equally into two new 150 cm^2 Falcon Cell Culture Flasks. 25ml of fresh cell medium supplemented with IL-2 was added to each flask and the flasks incubated for a further 72 hours.

Glutamine removal

To determine to what extent the level of glutamine in the cell medium had on CD4+ T lymphocyte activation Dynabead CD3/CD28 stimulation beads (Invitrogen) were used to stimulate CD4+ T lymphocytes in serial dilutions of glutamine ranging from 0.1 to 10mM. The FBS (fetal bovine serum) was dialyzed with a "Slide-A-Lyzer" Dialysis Cassette (30ml; 3.5K MWCO; Thermo Scientific) to remove small molecules including glutamine *(for procedures see Appendix A).* Then 100ul of a CD4+ T lymphocyte population ($15x10^6$ cells/ml) in cell medium (DM5030: 10% dialyzed, inactivated FBS (fetal bovine serum); 0.5% Penicillin-Streptomycin; IL-2 30U/ml; 36.5°C) were distributed in a 96-well curve-bottomed microplate and activated with 18.75ul Dynabeads CD3/CD28 Expansion beads ($4x10^7$ beads/ml; Invitrogen) per well *(washed first, as described earlier)*. The microplate was then incubated at 36.5°C for 48 hours. 25ul of 3H-Thymidine (40 uCi/mL; Nerliens) was then added to each well and then incubated for a further 24 hours. Cells were then harvested and incorporation of 3H-thymidine was measured using a scintillation counter to determine rate of proliferation. All tests were performed in triplicate.

Glutaminase inhibition – CD4+ T Lymphocytes

Two potential compounds were evaluated to disrupt cellular glutamine metabolism in CD4+ T lymphocytes at the level of Glutaminase *(see Glutaminase Inhibitors).* An assay was set up to test whether introduction of these inhibitors would have an inhibitory effect on proliferation as had been observed in other highly proliferative cells. The assay protocol was identical to the *glutamine removal* method described in the section above (minus the dialysing step, and supplementing the medium with 500uM L-glutamine). In addition both compound 968 (SPECS) and BPTES (Drug Discovery Laboratory AS) were included in the assay. Due to precipitation issues both inhibitor stocks were made with 100% DMSO. Serial dilutions of both inhibitors were made so that final assay concentrations were 0.1, 1, 10 and 100uM after addition of 0.5ul per well (max 0.5% DMSO per well). Control wells (minus inhibitor, with DMSO) were included.

3H-thymidine incorporation was measured after 72 hours. All tests were performed in triplicate.

2.5 qPCR – Gene Expression

Quantitative polymerase chain reaction (qPCR) was used to detect the gene expression levels in the naïve and activated CD4+ lymphocyte samples. RNA was first extracted from target samples and then converted to cDNA using reverse-transcriptase reaction. The cDNA templates were then exposed to gene specific probes and analysed using qPCR to determine relative expression levels.

RNA isolation

The RNeasy Plus Mini Kit (Qiagen) was used for RNA isolation from the naïve and activated CD4+ lymphocyte samples. All work was performed on ice. A buffer containing 1 part β-mercaptoethanol to 100 parts RLT Plus buffer (from RNeasy Kit) was added to each sample (350uL per sample). The RLT Plus buffer contains highly denaturing guanidine-isothiocyanate mix which inactivates RNases and ensures purification of intact RNA. The CD4+ lysate/buffer solution was then homogenized with a syringe and needle (20 gauge/0.9mm diameter; approx. 10 cycles was sufficient). The homogenized solution was then spun through the gDNA Eliminator columns (30 seconds at 10000rpm), which in combination with the high-salt RTS buffer removes genomic DNA. 350uL of 70% ethanol was then added to the flow-through which was then transferred to an RNeasy spin column and centrifuged for 15 seconds at 10000RPM. During centrifugation the total RNA binds to the internal membrane and any impurities are washed away. The flow-through was then discarded and 700uL RW1 solution (from RNeasy kit) was added to the RNeasy spin column and then centrifuged for a further 15 seconds at 10000RPM. The flow through was discarded again. 500uL RPE solution (from RNeasy kit) was then added to the spin column and centrifuged for 15 seconds at 10000RPM, after which the flow through was discarded once more. This process was repeated twice to rinse any

contaminants from the RNA collection membrane. The column was then placed inside a supplied 1.5mL collection tube and 50uL of 60 °C RNA-free water was added directly to the membrane to elute the RNA. The RNeasy Spin Column and collection tube was then jointly centrifuged at 10000RPM for 1 minute to collect the purified RNA.

RNA concentration

The NanoDrop 2000c Spectrophotometer (Thermo Scientific) was used to measure total RNA concentrations in the activated and non-activated CD4+ T lymphocytes. The program was set to "Group: Classic" and "Nucleic Acid" in the home screen and to "RNA" analysis at the measurement screen. First 2uL of the RNase-free water was pipetted onto the measurement pedestal of the Nanodrop 2000c and a BLANK reading initiated from the measurement screen. The pedestal arm was then raised and the sample wiped from the upper and lower pedestals using a lint-free laboratory wipe. Next, 1-2uL of the purified RNA solution was pipetted onto the lower pedestal, the sample named, and a measurement initiated. The process was repeated for all samples and the data exported to Excel. All work was performed on ice. The RNA concentrations were standardized for each sample with the addition of RNase-free water to the higher concentrates. *(see Appendix C for a complete list of total RNA concentrations)*.

Reverse Transcription

Complementary DNA (cDNA) synthesis from the purified RNA samples was achieved using the QuantiTect Reverse Transcription Kit (Qiagen). Reverse transcriptase enzymes are multifunctional, with 3 distinct enzymatic activates: an RNA-dependent DNA polymerase and DNA-dependent DNA polymerase transcribes cDNA from the RNA template; and an exoribonuclease then degrades the RNA. These 3 steps allow for transcription of RNA into DNA for later detection. The kit provides a two-step process that: 1) eliminates genomic DNA and; 2) synthesizes cDNA using a reverse transcriptase reaction. All work was performed on ice.

1. Genomic DNA removal:

The genomic DNA elimination reaction was performed according to Table 3.

Table 3. *Genomic DNA elimination reaction components*

Component	Volume/reaction	Final Concentration
gDNA Wipeout Buffer, 7x	2ul	1x
purified CD4+ RNA	(from Nanodrop 2000c)	
RNase-free water	Variable	
Total volume	**14ul**	**-**

The elimination reaction was then incubated for 2 minutes at 42 °C and then placed back on ice.

2. Reverse transcription:

The reverse transcription master mix was prepared according to Table 4.

Table 4. *Reverse-transcription reaction components.*

Component	Volume/reaction	Final Concentration
Reverse-transcription master mix		
Quantiscript Reverse Transcriptase	1ul	
Quantiscript RT Buffer	4ul	1x
RT Primer mix	1ul	
Template RNA		
Entire gDNA elimination reaction	14ul	
Total volume	**20ul**	**-**

Fourteen uL of the RNA template mix from the previous step is added to each PCR tube containing the reverse-transcription master mix and incubated in a thermal cycler for 15 minutes at 42 °C; a further3 minutes at 95 °C and then at 4 °C before being placed immediately on ice. A negative control (no reverse transcriptase; no-RT) was included to test for any contaminating genomic DNA. This is done by performing a control reaction with no reverse transcription added.

qPCR

Quantitative polymerase chain reaction (qPCR) and TaqMan Gene Expression Assays (Applied Biosystems) were used to amplify and detect the specific nucleic acid sequences used to synthesise the three Glutaminase isoenzymes in the stimulated and non-stimulated CD4+ lymphocyte samples. The TaqMan assays rely on the use of sequence-specific reporter probes

that emit a fluorescent signal when cleaved from the cDNA template during thermal cycling and the transcriptive action of DNA polymerase *(see figure 15).*

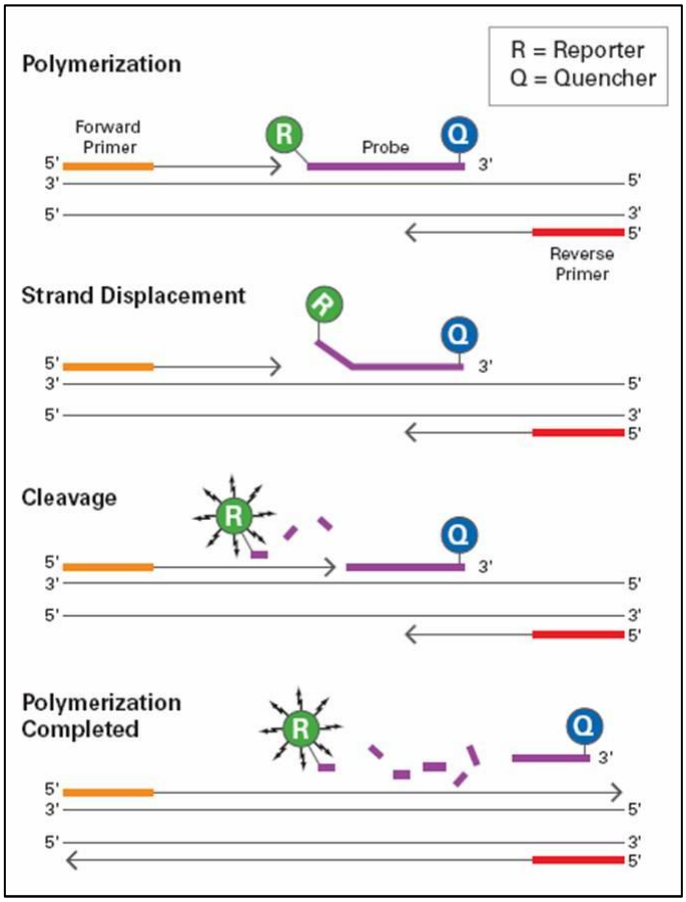

Figure 15. RT-PCR with fluorescent probes. *Polymerisation of the cDNA template cleaves the gene-specific TaqMan probe (purple) only if it is hybridized to the target sequence. The probe fragments and the reporter (R – green) displaces, separating from the quencher (Q – blue). The reporter emits a fluorescent signal which is detected by the RT-PCR equipment in real-time.* *(Image retrieved 20 March 2013, from: www SDS (sodium dodecyl sulphate).appliedbiosystems.com)*

After reverse transcription the TaqMan Gene Expression assay reagents were thawed on ice and combined with the cDNA templates in a 96 well PCR microplate according to Table 5 *(below)*. Purified water was added to each cDNA template if greater volumes were required.

Table 5. *qPCR gene expression reaction components*

Component	Volume/reaction	Final Concentration
TaqMan Master Mix PLUS		
TaqMan Assay (10:1)	11ul	2x
cDNA template	9ul	
Total volume	**20ul**	

The reaction mix was then centrifuged for 1 minute at 1200rpms and the microplate placed inside an Applied Biosystems 7900HT Fast Real-Time PCR system with the thermal cover attached. The system was set to determine *relative* DNA levels (ΔΔct (RQ)); detectors were added according to the plate setup and samples labelled. Thermal cycling was set as per Table 6.

***Table 6**. PCR Thermal cycling settings*

Step	Incubation	Enzyme activation	PCR	
	HOLD	HOLD	CYCLE (40 Cycles)	
			Denature	Anneal/Extend
Time	2 min	10 min	15 sec	1 min
Temperature	50 °C	95 °C	95 °C	60 °C

Thermal cycling took approximately 2 hours. Data was then analysed using the accompanying Applied Biosysetms Sequence Detection System (SDS) software (v2.3).

2.6 Glutamine flux – Mass Spectrometry

In an attempt to characterise the intracellular concentration and flux of glutamine in proliferating CD4+ T lymphocytes we collaborated with the Department of Medical Biochemistry at Oslo University Hospital. Mass spectrometry is a method for determining the composition of a sample; including the mass, chemical and electrical properties of the particles and molecules that make up the sample[62]. In a typical mass spectrometry measurement the sample (be it solid, liquid or gas) is first ionized then forced through an analyser comprising of electric and magnetic fields which affects the ionised sample's speed and direction according to its mass and charge. The ions pass into a detector which identifies and quantifies the analyte depending on its trajectory and latency.

Mass spectrometry sample preparation

On the seventh day of the *7 day expansion assay (described earlier under CD4+ T lymphocyte activation)* the expanding CD4+ T lymphocyte population was transferred to two 50ml Falcon Conical Tubes and centrifuged at 1500RPM for 7 minutes. The supernatant was removed and discarded and the cell pellets gently resuspended in 10ml of 36.5 °C PBS to wash the cells of the original cell medium. This step was repeated twice. The cells were then counted using the TC10 Cell Counter (Biorad) and then, depending on total yield, resuspended in a ^{13}C-glutamine medium (DM5030; ^{13}C-glutamine 500uM; Glucose 5mM; $NaHCO_3$ 3.7g/ml; 0.5% Penicillin-Streptomycin; 36.5°C) to allow for distribution of 150ul cell medium with approx. 750 000 CD+ lymphocytes into 1.5ml Eppendorf tubes. The tubes were kept in a 36.5 °C water bath to maintain optimum temperatures. Two holes were pierced in the Eppendorf tube lids. The tubes were then incubated for 0, 2, 4, 8 and 24 hours at 36.5°C. After incubation the tubes were centrifuged at high speed (15000RPM) for 10 seconds and the medium removed and saved in separate Eppendorf tubes. The cell pellet was washed with 300ul PBS then centrifuged again at 15000RPM for 10 seconds. This was repeated two times to remove the labelled glutamine medium. The cell pellets and the saved medium were then lysed with 450ul -80°C 80% MS-grade Methanol (Merck), left at room temperature for 10 minutes then centrifuged again at high speed for 1 minute. The supernatants of both samples were then transferred into fresh 1.5ml Eppendorf tubes and, along with the cell pellets, snap frozen in a -78°C Ethanol/dry ice solution and stored in a -80℃ freezer for mass spectrometry analysis.

This protocol was also used in an attempt to characterise the flux of glutamine into proliferating CD4+ T lymphocytes in the presence of the two Glutaminase inhibitors: compound 968 and BPTES. All parameters were identical. In addition, 0.75ul BPTES /968 (20mM stocks; 100% DMSO) was added to specific Eppendorf tubes directly before incubation to achieve a final concentration of 100uM. Samples were incubated once again for 0, 2, 4, 8 & 24 hours at 36.5°C and then processed for mass spectrometry in the procedure described above.

2.8 Glutaminase inhibition – protein

The effects of compound 968 and BPTES were also tested on an *in vitro* Glutaminase enzyme assay. Figure 16 *(below)* shows the two-step catabolism of glutamine to α-ketoglutarate and NADH in the presence of the enzymes Glutaminase (GLS) and glutamate dehydrogenase (GDH). Using a micro plate reader the appearance of NADH in the second reaction was quantified as a measurement of Glutaminase activity, alone and in the presence of the inhibitors. The effect of phosphate on Glutaminase activity was also measured.

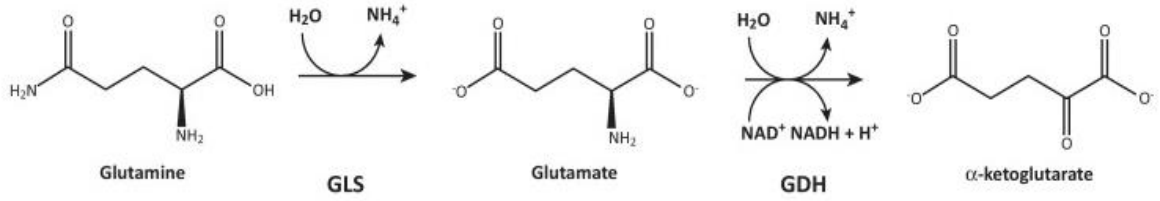

Figure 16. *Two step reaction to form alpha-ketoglutarate and NADH from glutamine through glutamate. GLS – Glutaminase; GDH – Glutamate Dehydrogenase.*

The first assay was performed as per Table 7 *(below)*. All components were prepared and stored on ice. 5 minutes before assay start they were removed from the ice and allowed to warm to room temperature.

Table 7. *Reaction components.*

Reaction mix (2x)		**Glutaminase mix (2.5x)**	
NAD (Roche)	4mM	Glutaminase**	(100mM, 1mM, 10nM)
GDH*	6 units	TRIS-acetate	50mM
L-glutamine	8mM		
TRIS-acetate buffer	50mM		
K_2PO_4	*100mM*		

* Glutamate Dehydrogenase (Sigma)
** *E. coli* (Sigma)

Five uL of 10% DMSO was added to a 96-well, flat-bottomed, clear microplate followed by 20ul of Glutaminase mix. Finally, 25ul of Reaction mix was added and the microplate placed in a Fluorostar OPTIMA microplate reader (BMG Labtech) which had previously been configured (96-well plate/Greinar BioOne; 50ul well volume; 340-10 Excitation filter; wavelength adjusted). All tests were performed in triplicates. Blank (50ul TRIS-acetate) and Negative controls (minus Glutaminase mix) were included. Blank corrected, raw and average data was exported to Excel. In the second assay the 5ul of 10% DMSO was substituted for 5ul from stock solutions of BPTES/968 (10% DMSO; 50mM TRIS-Acetate buffer) to achieve final concentrations of 0.1, 1, 10 & 100uM. No dipotassium phosphate was used in the second assay.

3.0 RESULTS

3.1 Characterisation of Glutaminase isoforms in naive CD4+ T cells

The isoforms of Glutaminase in CD4+ T cells are not well characterized. Therefore, we prepared western blots of protein extracts prepared from CD4+ T cells (Figure 16). Naïve mature CD4+ lymphocytes were isolated from buffy coats as described under *Methods*. Cells were lysed, diluted in an SDS sample buffer and total protein (15ug/lane) separated by SDS-PAGE in a 7.5% acrylamide gel. Fractionated proteins were transferred to PVDF membranes which were incubated with antibodies to the three known Glutaminase isoforms: KGA (250-1000:1), GAC (600:1) and LGA (500:1); in addition to a control, α-tubulin (5000:1). Figure 17 depicts KGA (73kDa) and to a lesser extent GAC (55kDa), but not LGA (66kDa), are expressed at detectable levels using this method in CD4+ T lymphocytes.

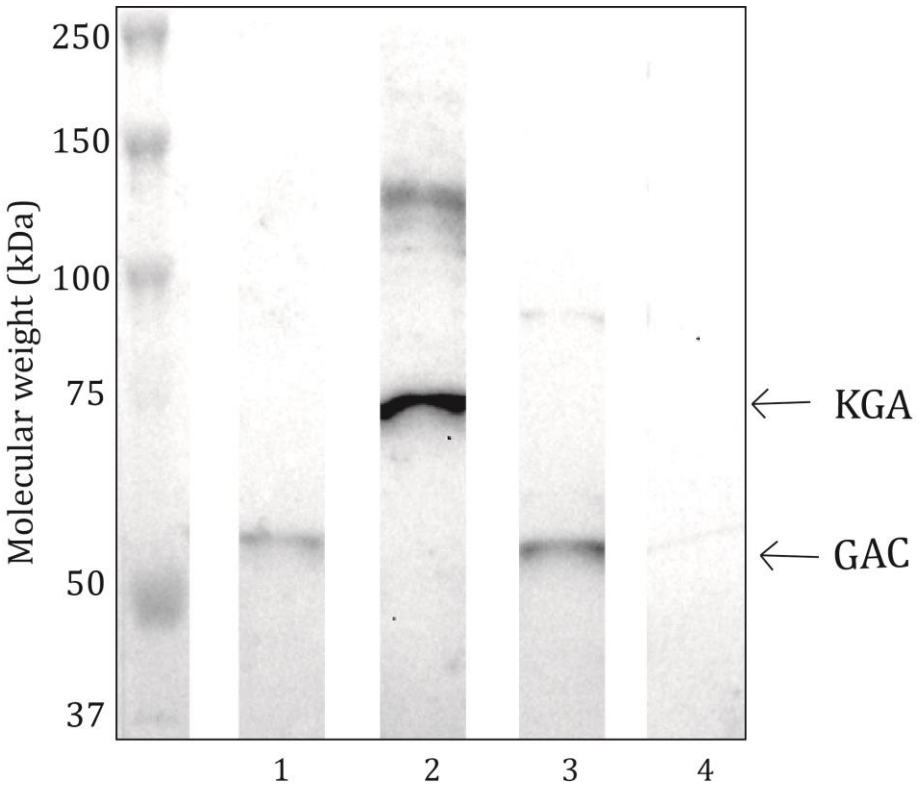

Figure 17. Relative Glutaminase isoenzyme levels in resting mature CD4+ lymphocytes. *Lane 1 - α-tubulin (55kDa); Lane 2 - KGA (arrow; 73kDa); Lane 3 - GAC (arrow; 55kDa); Lane 4 – LGA (not visible).*

3.3 Characterisation of Glutaminase isoforms in activated CD4+ T cells

Since expression of the KGA and GAC Glutaminase isoforms was evident in naïve CD4+ T lymphocytes, we next investigated if the same set of Glutaminase isoforms were expressed in CD4+ T lymphocytes activated through their CD3 and CD28 cell surface markers.

CD4+ T lymphocytes were activated as described under *Methods*. Figure 18 depicts that within minutes of the addition of the anti-CD3 and anti-CD28 coated beads, CD4+ lymphocytes begin to surround individual beads *(photos C & D).* A successful stimulation was evident from the yellow medium and "budding" of the activated T cells; and the relative homogenous cell dispersion & cell morphology, and pink medium of the non-stimulated wells *(A & B).* At 72 hours the activated cells have conglomerated around beads and swelled in size *(E & F).*

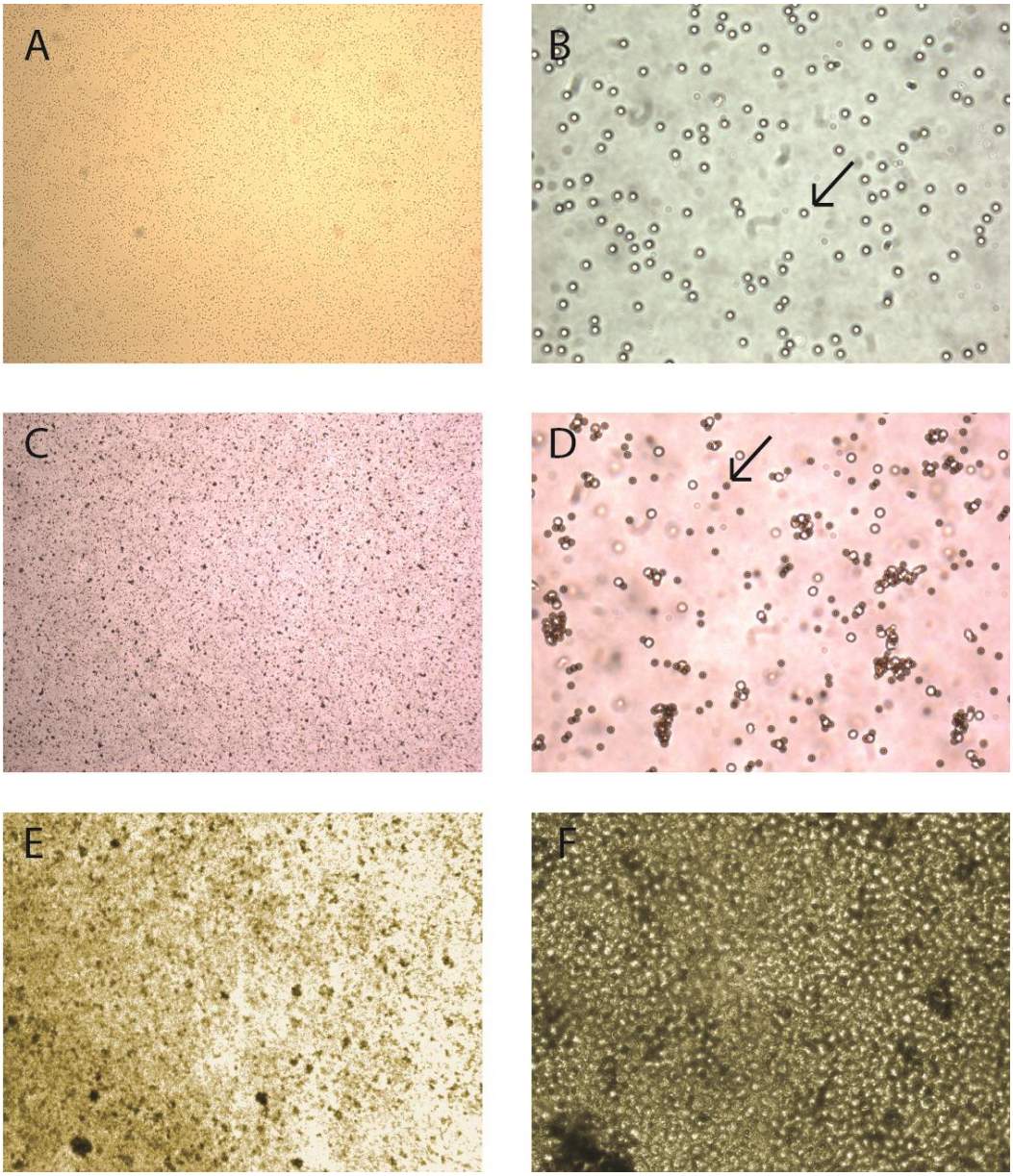

Figure 18. Images of naïve and activated CD4+T lymphocytes in cell culture. A/B: *Naive CD4+ lymphocytes (4x)/(40x)- the arrow on the left indicates a single CD4+ T lymphocyte;* ***C/D****: CD4+ lymphocytes stimulated with Dynal beads (dark-arrow) after 2 min incubation(4x)/(40x) – the arrow indicates a single Dynal bead;* ***E/F****: Stimulated CD4+ lymphocytes after 72 hours incubation time (4x)/(40x).*

To confirm that the budding and morphological changes of the CD4+ cells seen in the above photos were a result of CD3/CD28 activation a solution containing a radioactive nucleoside, 3H-thymidine, was added to the cell medium after 48 hours. The rate of 3H-thymidine incorporation into DNA of the CD4+ T cells was then measured after 24 hours using a scintillation counter.

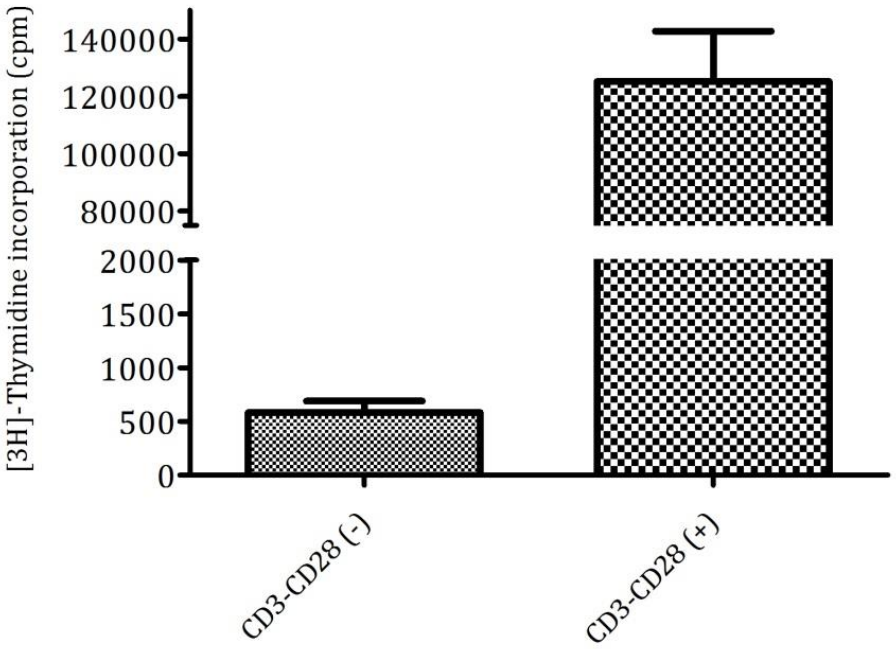

Figure 19. *3H-thymidine nucleoside incorporation into newly synthesized DNA in stimulated and non-stimulated CD4+ lymphocytes. Radioactivity measured in counts per minute (cpm).*

Figure 19 demonstrates that after addition of the CD3/CD28 activation beads the rate of 3H-thymidine uptake and incorporation into newly synthesized DNA over the 24 hours increased from approximately 600 (+/- 60) cpm to 125,000 (+/-10,000) cpm confirming successful activation.

To determine the relative expression of the three Glutaminase isoenzymes in CD3/CD28 activated CD4+ T cells we again prepared samples and separated total protein in the manner described previously *(and in more detail in Methods).* Figure 20 demonstrates that after CD3/CD28 activation there appears to be a considerable drop in the relative level of the KGA Glutaminase isoform (73kDa) and a concurrent increase in the level of the GAC isoform (55kDa). This was accompanied by the appearance of non-specific banding on both the KGA blot (approx. 48kDa) and GAC blot (approx. 42kDa) that were not present in the non-activated blots. Once again the LGA isoform was not detected.

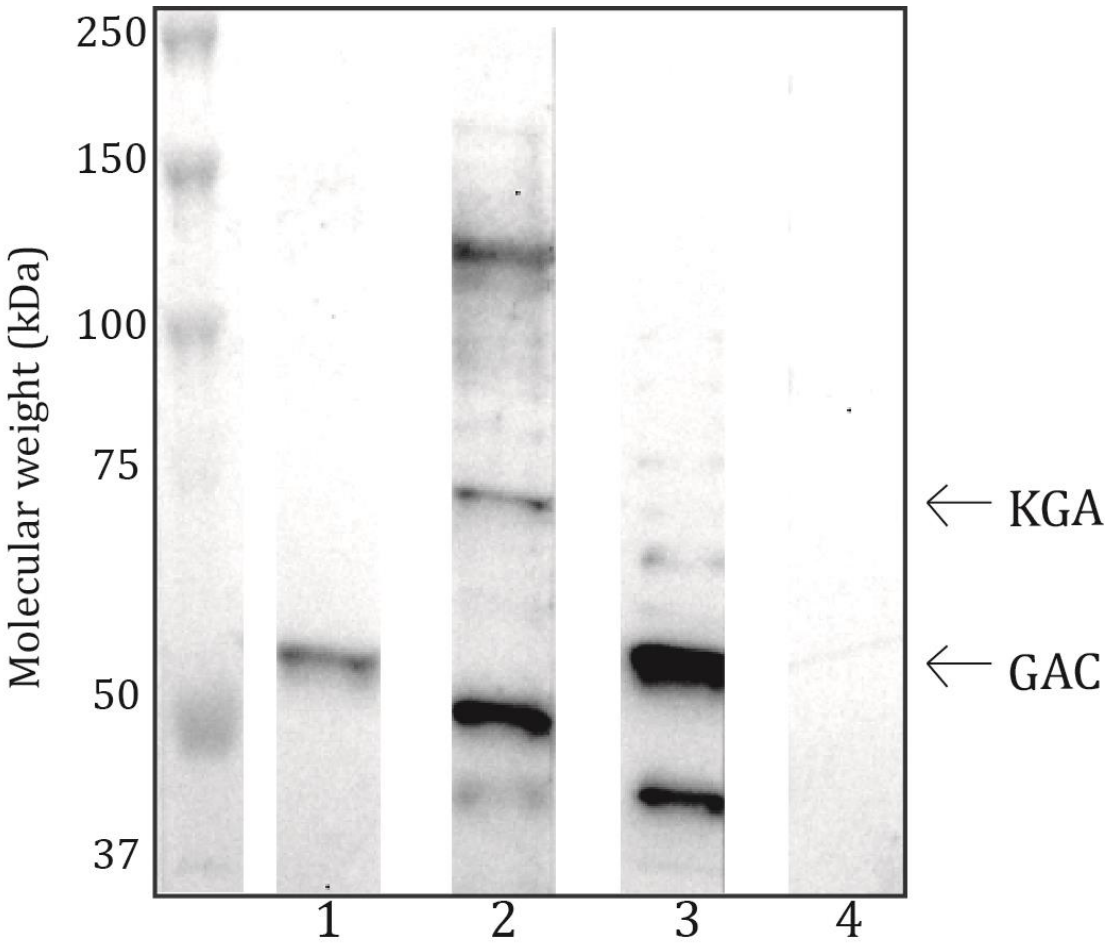

Figure 20. *Relative Glutaminase isoenzyme levels in CD3/CD28-activated CD4+ lymphocytes. Lane 1 - α-tubulin (55kDa); Lane 2 - KGA (arrow; 73kDa); Lane 3 - GAC (arrow; 55kDa); Lane 4 – LGA (not visible).*

In an attempt to understand the dynamics of this change in relative isoenzymes expression between KGA and GAC we set up a time course assay that could examine the relative protein levels in 12 hour intervals for a total of 72 hours after activation. The procedure used was identical to the one described earlier. This demonstrated (as illustrated in Figure 21) that KGA levels drop rapidly in the first 12 hours after CD4+ T lymphocyte activation. Concurrently, there is a slightly delayed increase in the relative concentration of the GAC isoform which reversed after 24 hours. This decrease in GAC concentration was not consistent with the previous blot where the presence of an intense band indicated high GAC levels at 72 hours.

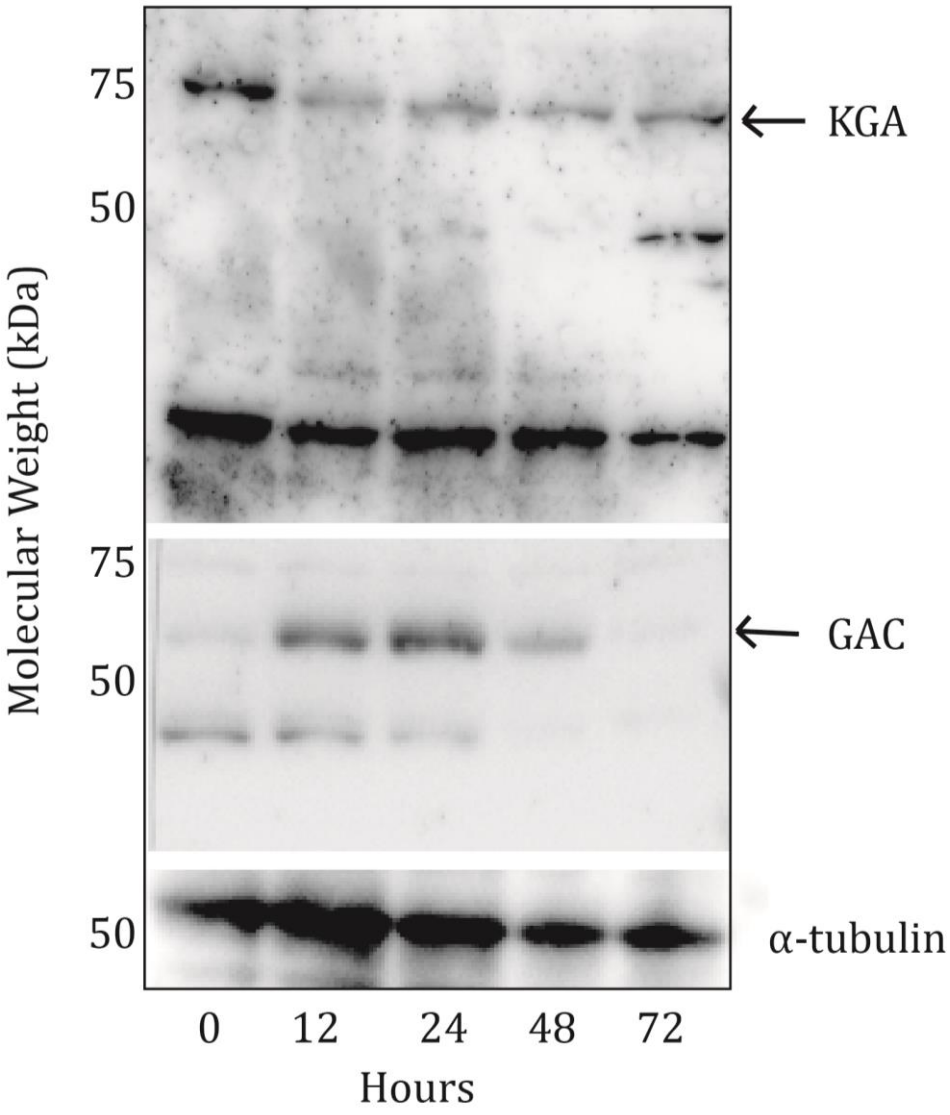

Figure 21. *Relative Glutaminase isoenzyme concentration at rest (0 hours) and at 12 hour intervals after CD3/CD28 activation (total 72 hours). Upper box - KGA (73kDa); Middle box – GAC (55kDa); Lower box - α-tubulin (55kDa).*

3.3 qPCR – Gene Expression

Our results showed elevated levels of the GAC Glutaminase isoform and a concurrent decrease in the KGA isoform in CD4+ t lymphocytes activated via their CD3 and CD28 surface markers. Next we wanted to determine whether this dynamic would be evident at the genetic level.

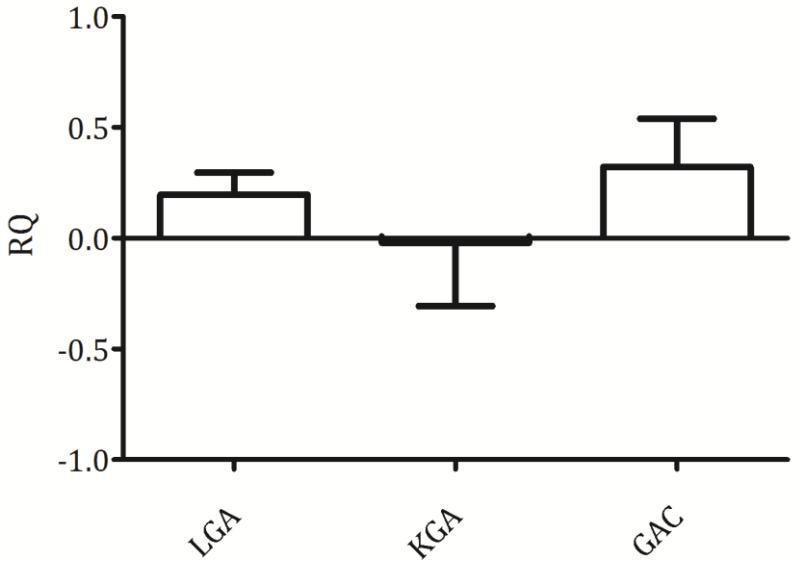

Figure 22. *Relative mRNA concentrations of Glutaminase isoenzymes in proliferating CD4+ lymphocytes compared to naïve cells. RQ – Relative Quantification.*

Total RNA was isolated from the CD4+ T cell samples and used to synthesize complementary DNA using reverse transcription. Each cDNA template was then probed three times: once for each of the LGA, KGA & GAC Glutaminase isoenzymes and amplified using real-time polymerase chain reaction (RT-PCR) *(see Methods)*. Figure 22 indicates a slight increase in relative concentrations of mRNA coding for the GAC protein; a weaker increase for the LGA isoform; and no relative change for the KGA form in CD3/CD28-activated CD4+ lymphocytes relative to naïve cells.

3.5 Glutamine removal

According to the literature, cancer cell transformation is associated with what has been described as "glutamine addiction"[47]. Based on this we wanted to determine how the removal of glutamine from the cell medium would affect the growth of activated CD4+ T lymphocytes.

Stimulation of the CD4+ lymphocytes was achieved in a manner described earlier and under *Methods.* Cells were incubated in a 96-well plate for 48 hours in cell mediums with varying concentrations of glutamine (0.01 – 10uM). Twenty five uL of 3H-thymidine was then added to

each well and allowed to incubate for a further 24 hours. 3H-thymidine incorporation was then measured using a scintillation counter to determine the rate of growth.

Results showed that reducing the concentration of glutamine in the cell growth medium below 1mM had a profound impact on CD4+ T cell proliferation, despite the availability of glucose *(see Figure 23).* Proliferation was shown to be stable in glutamine concentrations higher than 1mM, with a subsequent sharp decline between 1 and 0.1mM. It appeared that at glutamine concentrations below 0.1uM cells proliferation was almost completely arrested.

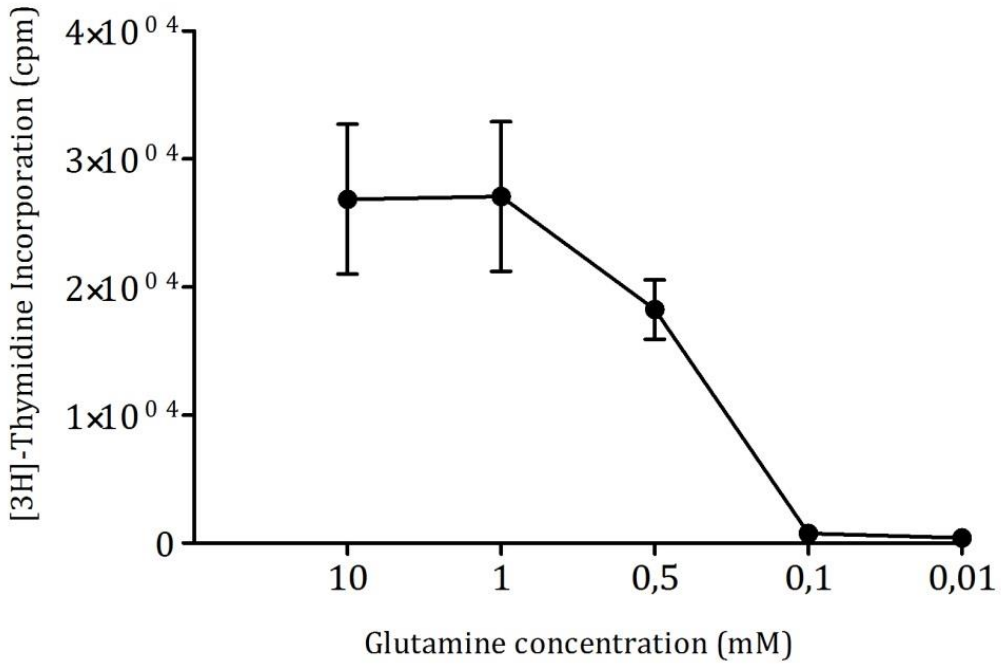

Figure 23. *CD3/CD28-activated CD4+ T lymphocyte 3H-thymidine incorporation in growth medium with limiting glutamine concentrations. Radioactivity measured in counts per minute (cpm).*

3.6 Glutaminase inhibition – CD4+ T Lymphocytes

Our results demonstrated that activated CD4+ lymphocytes are dependent on glutamine to proliferate. Next we wanted to determine if blocking glutamine metabolism would have a similar effect on activated CD4+ T lymphocytes.

CD4+ T lymphocytes were activated via their CD3/CD28 surface markers by magnetic beads and incubated in a standard growth medium as described in detail in *Methods.* In addition, two known Glutaminase inhibitors were added to the medium: compound 938 and BPTES (detailed in *Introduction)*. 3H-thymidine was added to each well after 48 hours and rate of incorporation was measured after a further 24 hours by a scintillation counter. Our results, as shown in Figure 24, demonstrate that the introduction of the Glutaminase inhibitors had a noticeable impact on 3H-thymidine incorporation at concentrations higher than 1uM: >50% reduction at 10uM and almost complete reduction (Compound 968) at 100uM ($p<0.05$). Interestingly, a slight increase in proliferation was observed in BPTES-inhibited wells above that of the stimulated cells without inhibitors.

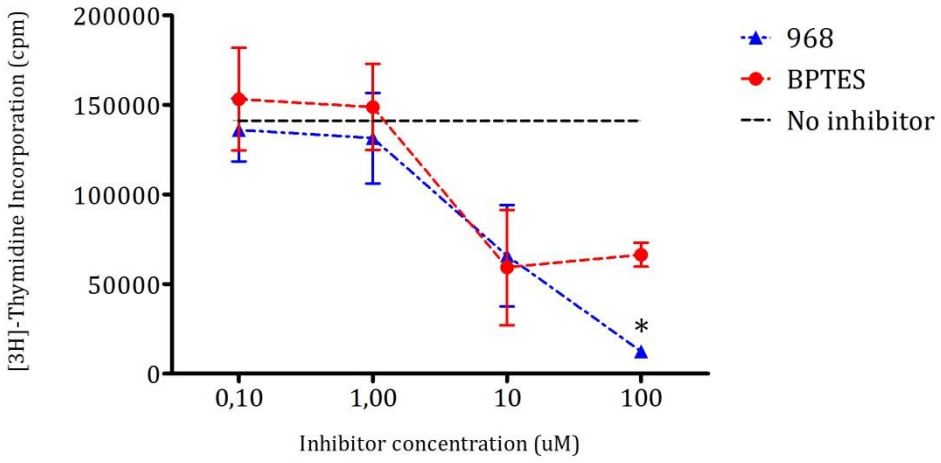

Figure 24. *[3H]-Thymidine incorporation of CD3/CD28-activated CD4+ lymphocytes in the presence of Glutaminase inhibitors 968 & BPTES. Radioactivity measured in counts per minute (cpm).*

3.7 Glutaminase inhibition - protein

The ability of activated CD4+ T lymphocytes to synthesize new biomass appeared to be significantly reduced through the introduction of Glutaminase inhibitors. We wanted to confirm that the two Glutaminase inhibitors were actually inhibiting Glutaminase, and that the results seen above were not a result of some other phenomenon.

First, activity of Glutaminase derived from *Escherichia Coli* was quantified by measuring the appearance of NADH generated in the second reaction of the catabolism of glutamine to α-ketoglutarate *(see Methods)*. In a 96 well clear-bottomed microplate a solution containing NAD+, Glutamate Dehydrogenase and glutamine in a TRIS-acetate buffer was mixed with a solution of Glutaminase *(see Methods for more details)*. A phosphate mixture was also included to determine its effect on Glutaminase activity. NADH appearance was measured using a microplate reader and the absorbance of UV light at 340nm.

Figure 25 depicts a dose-response relationship between the concentration of Glutaminase and the appearance of NADH in our tests. Our results also showed that in the presence of phosphate the conversion of glutamine to α-ketoglutarate appears to be attenuated.

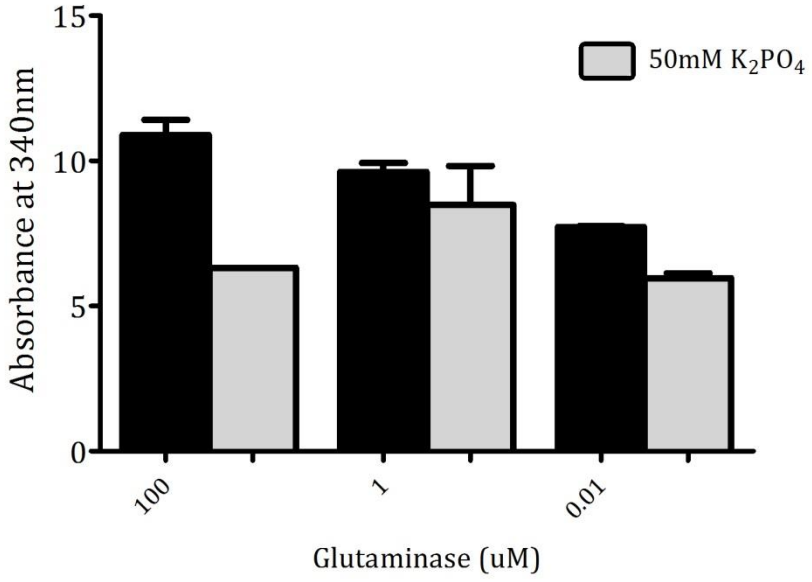

Figure 25. NADH appearance as a measurement of Glutaminase activity. *NADH concentration measured by UV absorbance at 340nm. Grey bars - 50mM K_2PO_4; Black bars – phosphate free. Glutaminase derived from* E. coli.

Next, compound 968 and BPTES were added in serial dilutions (0.1 – 100uM) to the reaction mixture described above. Once again NADH appearance through 340nm UV absorption was used to determine Glutaminase activity.

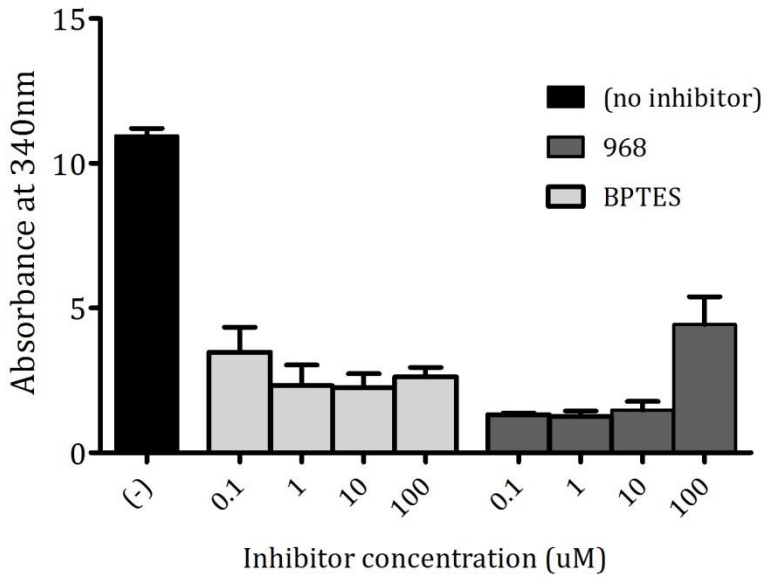

Figure 26. NADH appearance as a measurement of Glutaminase activity in the presence of inhibitors: 968 and BPTES. *NADH concentration measured by UV absorbance at 340nm. Black bar – no inhibitor; light grey – BPTES; dark grey – 968. Glutaminase derived from* E. coli.

Together these experiments demonstrated that Glutaminase activity is powerfully inhibited by both inhibitors *in vitro* at all concentrations by up to 90% *(Figure 26).*

3.4 Glutamine flux – Mass Spectrometry

Based on the results depicted in figures 22-26, we suggest that T cell proliferation induced through the TCR/CD3-CD28 complex requires the presence of glutamine. Therefore, we wanted to characterise the intracellular concentration, and flux of extracellular added, glutamine in proliferating CD4+ T lymphocytes with and without the use of the two Glutaminase inhibitors.

Proliferating CD4+ T cells were generated through a 7-day extended version of the CD4+ T cell activation protocol explained in detail in *Methods.* After 7 days the cells were washed free of the growth medium and then incubated for up to 24 hours in a 500uM ^{13}C-glutamine-labelled cell medium. The medium and the cell pellet where then lysed with 80% methanol and then analysed using mass spectrometry to determine ^{13}C-glutamine concentrations. Figure 27 clearly

depicts a steady reduction (up to 60% after 24 hours) in the amount of labelled-glutamine present in the cell medium during the measurement period (0-24 hours).

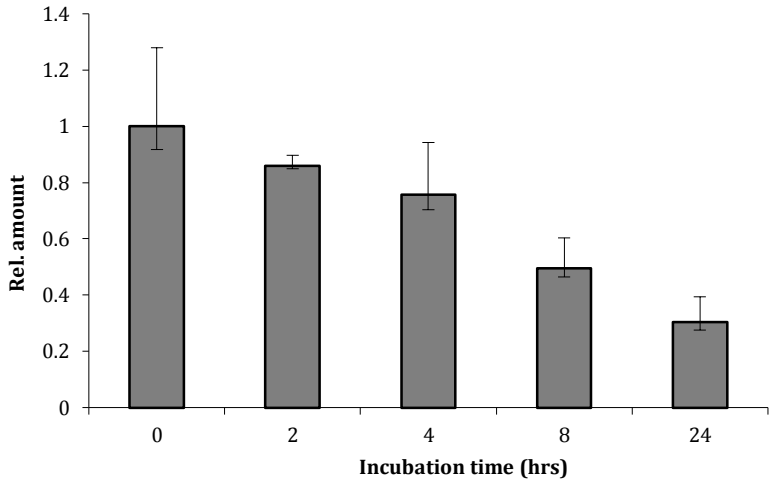

Figure 27. Glutamine consumption from cell medium containing proliferating CD4+ T lymphocytes. *Grey bars depict relative medium glutamine concentrations in intervals over 24 hours.*

To investigate if the reduction in media glutamine was associated with Glutaminase activity we added 968 and BPTES and measured glutamine levels over a time course of 0 to 24 hours.

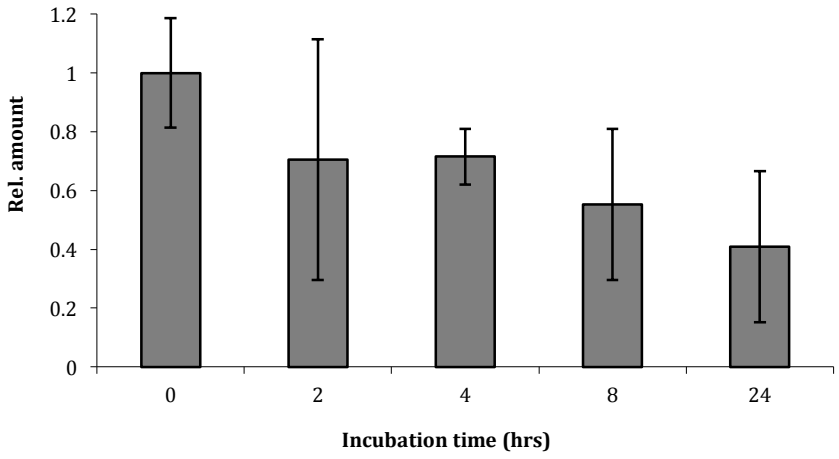

Figure 28. Glutamine consumption from cell medium containing BPTES and a CD4+ T lymphocyte population*. Grey bars depict relative medium glutamine concentrations in intervals over 24 hours.*

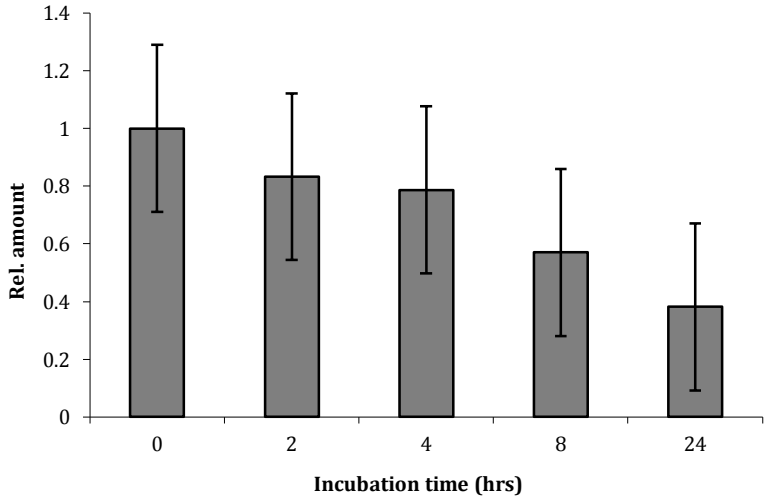

Figure 29. Glutamine consumption from cell medium containing 968 and a CD4+ T lymphocyte population. *Grey bars depict relative medium glutamine concentrations in intervals over 24 hours.*

This demonstrated that glutamine reduction in the media was comparably reduced regardless of the presence of inhibitors. Figures 28 and 29 *(above)* show similar patterns of glutamine reduction from the cell medium.

To investigate if glutamine was taken up by the T cells we measured relative intracellular concentrations of glutamine up to 24 hours in the presence and absence of the glutamine inhibitors BPTES and 968 *(see figures 30, 31 and 32).*

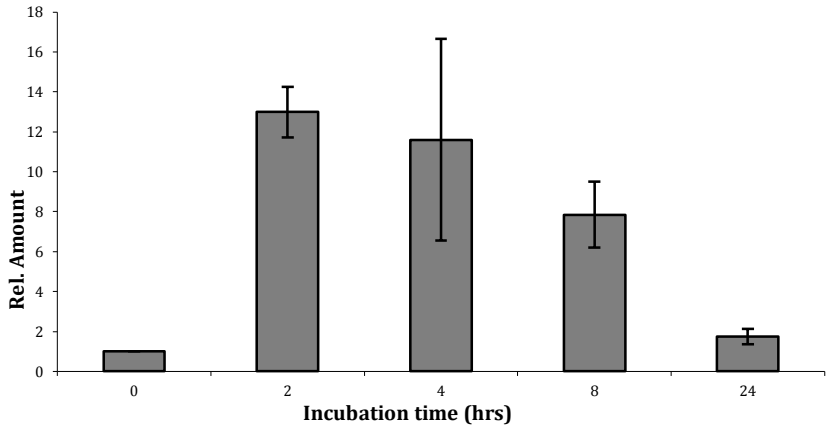

Figure 30. Relative intracellular concentrations of glutamine in proliferating CD4+ lymphocytes in the presence of the Glutaminase inhibitor, BPTES. *Grey bars depict relative medium glutamine concentrations in intervals over 24 hours.*

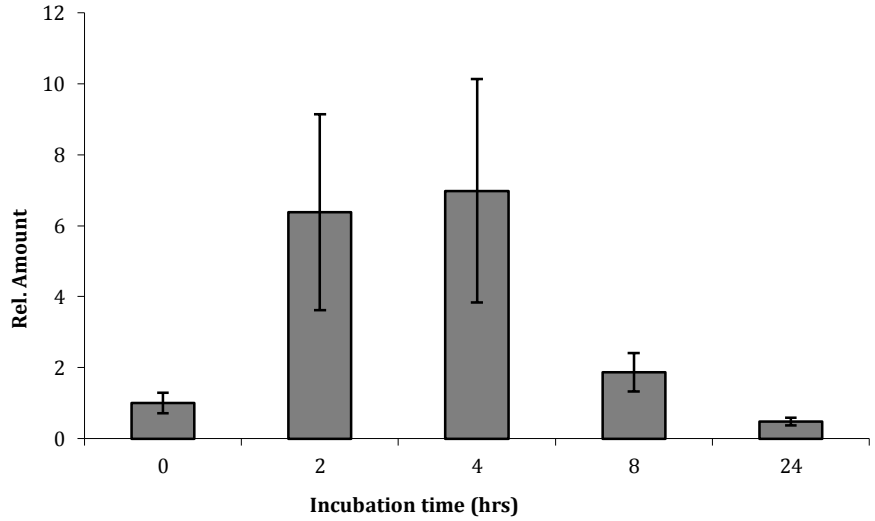

Figure 31. Relative intracellular concentrations of glutamine in proliferating CD4+ lymphocytes in the presence of the Glutaminase inhibitor, 968. *Grey bars depict relative medium glutamine concentrations in intervals over 24 hours.*

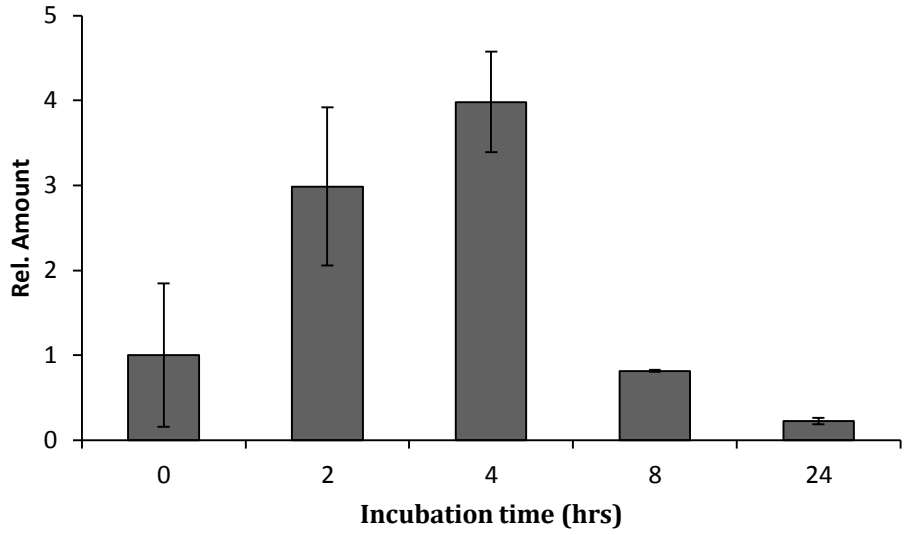

Figure 32. Relative intracellular concentrations of glutamine in proliferating CD4+ minus Glutaminase inhibitors. *Grey bars depict relative medium glutamine concentrations in intervals over 24 hours.*

Finally, a regression analysis was performed on the absolute values to determine the statistical relationship between the three assays.

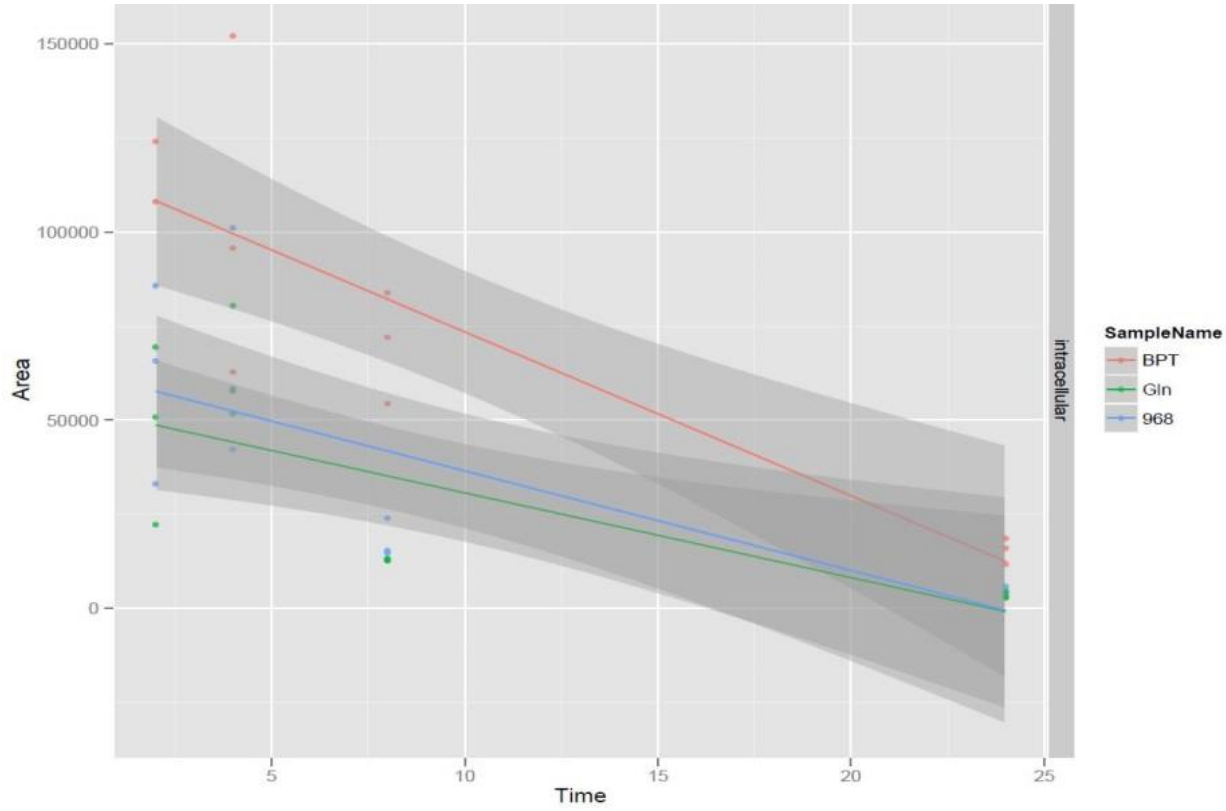

Figure 33. A regression analysis of absolute intracellular glutamine levels in the three MS assays. *Red – BPTES, Blue – 968, Green – no inhibitor. Grey area denotes 95% confidence percentile. Time = hours from assay start.*

Figures 30, 31 and 32 demonstrate that the relative levels of intracellular glutamine in CD4+ T lymphocytes increase rapidly over the first 4 hours then gradually decline to baseline in a similar manner regardless of the presence of the Glutaminase inhibitors. The BPTES assay showed the biggest increase (12 times baseline at 2 hours), while the 968 and non-inhibitor assays showed similar values. This was reflected in the regression analysis of the absolute values: There was no statistical difference in intracellular glutamine concentrations between the assay with no inhibitor and that containing compound 968. There was, however, a significant increase in the amount of intracellular glutamine in the BPTES assay between hours 0 and 8. After 24 hours incubation this statistical difference vanished.

4.0 DISCUSSION

Cancer cells exhibit up-regulated glycolytic activity and rapid turnover of biosynthetic precursors compared with somatic cells. This proliferative phenotype is common between many different proliferating cells. This relationship allowed us to hypothesize whether the "glutamine addiction" seen in cancer cells was applicable to proliferating human lymphocytes, and if, ultimately, we could disrupt glutamine metabolism and attenuate proliferation.

Glutaminase

The up-regulation of the GAC Glutaminase isoform in activated CD4+ lymphocytes seen in our Western Blot analyses is consistent with similar studies on colorectal tumours[63], gliomas[64], central nervous system tumours[65] and breast tumours[66]. Also compatible is the inconsistency between the increase in Glutaminase protein and expression of the *gls* gene[60;63;64]. Our results also hinted at a slight increase in mRNA levels of GAC, but the data was not significant and exhibited wide variance as to be unreliable. However, previous studies have demonstrated elevated GAC mRNA levels in colorectal carcinomas[63], breast tumours[66] and glyomas[64], with concurrent increases in GAC protein concentration. However, a previous study by Szeliga, et al. demonstrated a 20 times increase in the level of LGA mRNA in neoplastic cells with no increase in protein concentration[67]. This may suggest that there is poor correlation between mRNA and protein for GLS, at least for certain isoforms.

The transcription factor myc up-regulates glutamine transporter synthesis and expression, increases mitochondrial glutaminolysis, and sensitizes many proliferating cells to glutamine withdrawal[35;68;69]. Interestingly, up-regulation of *myc* also corresponds to increased GAC expression in B lymphoma and prostate cancer cells[49]. In addition, GAC was the only Glutaminase isoform located in the mitochondria of lung, breast and prostate cancer cells[60]. Combined, this suggests that the expression of the three Glutaminase isoforms may not be

under direct genetic control, but that *myc*, and perhaps other, undiscovered pathways, may, indirectly, control their expression and activity through post-translational modifications. The presence of sub-bands in our western blots taken from activated T lymphocytes may be evidence of this. It also suggests that the up-regulated GAC isoform may offer neoplastic cells a selective advantage under proliferation due to its exclusive location in the mitochondria[60].

The kinetics of the three Glutaminase isoforms, particularly GAC, in the presence of phosphate may also hint at another selective advantage for T lymphocytes under proliferation. Unfortunately we were unable to test the human Glutaminase isoforms. However, our kinetic data demonstrated that in the presence of 50mM of phosphate the activity of Glutaminase derived from *E. coli* was reduced. This was consistent with other experiments[70]. A comparison with the mammalian Glutaminase isoforms illustrates the widely differing phosphate-dependent characteristics that exist between species, and also between isoforms of the same species. The mammalian GAC isoform, for example, is massively up regulated (40x) in solutions containing 50mM phosphate whilst the LGA variant is virtually unresponsive[60]. This suggests that phosphate is required for optimal catalytic efficiency in the human GAC Glutaminase isoform, which our results indicate is the dominant isoform in proliferating T cells. We were unable to find any data regarding mitochondrial phosphate concentrations in proliferating lymphocytes. To what extent an increase in mitochondrial phosphate concentration contributes to the activation/up-regulation of GAC Glutaminase seen in proliferating T cells remains to be seen.

Glutamine dependence

A dependence of activated T cells on sufficient extracellular glutamine was witnessed in our 3H-thymidine incorporation assays. Initially, the cells that were completely deprived of glutamine exhibited the same proliferative rate as those with plentiful glutamine. This led us to speculate at whether the glutamine concentration or other crucial factors in the fetal bovine serum (FBS) used in the cell medium was negating any effects of the glutamine removal. Once we had

dialyzed the FBS and removed all small molecules below 3500 kDa, including glutamine, we immediately got results. We found that at glutamine concentrations lower than 0.5mM the proliferative ability of CD4+ lymphocytes was reduced by approx. 50%; and at 0.1mM, almost 100%. This dependency was backed up by our spectral analyses of cell media containing proliferating T lymphocytes. Over a 24 hour period the extracellular glutamine concentration was reduced by over 60%. This rapid glutamine consumption can be seen in many cancer cells, with certain tumours consuming so much glutamine they sink plasma glutamine levels[67;69]. Together this suggests that proliferating CD4+ T lymphocytes, as with many other highly proliferative cells, are absolutely dependent on adequate extracellular glutamine. Taken one step further it may be suggested that the ability of the immune system as a whole is dependent on glutamine. If this is the case, it may have significant implications from a clinical perspective when we consider how best to treat diseases that are known to deplete plasma glutamine levels including chronic inflammatory diseases, sepsis, severe burns and malnutrition[71].

We have shown that the expression of a highly catalytic glutamine isoform is up-regulated in proliferating CD4+ T cells. In addition, elevated glutamine activity features in many different tumour types[57;59;67]. When we consider glutamine's role as a precursor in the metabolic and biosynthetic requirements of these cells, it is perhaps of little surprise that intracellular glutamine concentrations are low to non-existent[72-74]. This suggests that glutamine is immediately catabolised once it enters the proliferating cell. However, our spectral analyses examining the intracellular glutamine concentration of proliferating CD4+ T cells did not reflect this. First, the small concentration of intracellular glutamine witnessed at 0 hours may be a result of the small delay in freezing/lysing the samples, therefore giving the cells a small window of opportunity to take up glutamine from the media. Despite this, a large increase in intracellular glutamine concentration peaked at four hours. An explanation for this could, in part, be explained by the 30-45 minute washing and distribution process performed before the cells are suspended in the labelled-glutamine medium. The washing medium is free of

glutamine (and other amino acids). Glutamine depletion is known to down-regulate glutamine transporters[75] – this may have had the effect of dampening the glutaminolytic machinery inside the cell. Hence, after glutamine addition and when the transporters up-regulated again, glutamine catabolism may have been delayed, thus the temporary increase in glutamine concentration. However, the same washing procedure had no effect on the consumption of glutamine from the cell medium seen in the other assays. It also seems unlikely given the slow nature (~12hrs) of the depletory effect[75]. Another consideration is the lack of IL-2 from our washing medium. IL-2 depletion has been shown to have an almost instant effect on nutrient uptake and metabolism in proliferating T lymphocytes[76], but once again this effect was not witnessed in the glutamine uptake experiments with the same procedures. If IL-2 depletion affected *only* the catabolism of glutamine and not the transporters (or there was a *delay* in its effect on the transporters) then this could explain the temporary peak in intracellular glutamine. Moreover, we saw a steady reduction of intracellular glutamine concentration after four hours incubation. Taken together with the steady uptake of glutamine from the cell medium, this suggests that there was an increase in glutamine catabolism after four hours. It is clear that our protocol would benefit from a more careful analysis of the signalling effects of IL-2 and how glutamine depletion affects the cells prior to assay start.

Glutaminase inhibitors

Recent research has highlighted Glutaminase as an exciting therapeutic target in tumours. Specifically, two allosteric Glutaminase inhibitors, compound 968 and BPTES have recently been used to slow the growth of glioma cells[77] and human breast tumours[57].

Glutamine intracellular concentration and flux

We exposed proliferating CD4+ T cells to both inhibitors and used Mass Spectrometry to analyse the glutamine flux from the cell medium, in addition to the intracellular glutamine concentration, over a 24 hour period. Our findings suggest that glutamine inhibition has little

effect on glutamine uptake in proliferating CD4+ T lymphocytes with almost identical clearance rates between the non-inhibited and inhibited cells. The pattern of glutamine appearance in the cells (increasing over the initial 2-4 hours and then sinking to baseline) between the three assays was very similar. Inhibitor/enzyme association is not instantaneous: the inhibitor must infiltrate the cell first and then bind to the enzyme. Our inhibitors were added to the cells at 0 hours and had no pre-incubation period. The observed delay in the increase of glutamine inside the cell may be a result of this. It is reasonable to suggest that pre-incubating the cells with the inhibitors could result in a much more rapid increase in intracellular glutamine. Interestingly, the intracellular glutamine concentration of the cells exposed to BPTES between 0-4 hours was significantly higher (2x) than the control cells and those exposed to 968. This suggests that the inhibitory effect of BPTES was greater than that of 968 - which appeared to have little effect. Why this significance gradually disappears over the 24 hours, however, is unclear. Indeed, the steady decline in glutamine concentration from both inhibitor assays is puzzling. Given the steady uptake of glutamine from the cells it may simply be of kinetic nature – the increasing glutamine concentration out-competes 968/BPTES for the binding seat on Glutaminase. Another interesting proposition is that of an alternate metabolic pathway for glutamine.

Enzyme inhibition

We exposed recombinant Glutaminase derived from *E. Coli* to both inhibitors and measured Glutaminase activity by the appearance of NADH. Due to the issues with our protocol detailed earlier, we were unable to obtain any convincing data. Our inability to obtain a dose-response relationship may, in part, be due to our references for inhibitor concentrations obtained from the literature (IC_{50} 0.14-19uM[78,57]). Given the structural differences between mammalian Glutaminase and Glutaminase from *E. Coli* a more thorough investigation into ideal inhibitor concentrations would be prudent. Regardless, we did see reduced catalytic activity of the *E. Coli* Glutaminase exposed to both inhibitors at all concentrations used (0.1 – 100uM). This and other kinetic analyses of recombinant human Glutaminase exposed to BPTES[58;78;79] or 968[57;60]

suggest that the reduced proliferation observed in tumour cells in the presence of these inhibitors *is* a result of Glutaminase inhibition.

CD4+ T lymphocyte inhibition

With this in mind we exposed CD3/CD28-activated CD4+ T lymphocytes to varying concentrations (0.1uM to 100uM) of both 968 and BPTES. At concentrations higher than 10uM, both inhibitors had a detrimental effect on cell proliferation. This is most likely due to conformational changes to mitochondrial Glutaminase and subsequent reduction in glutaminolysis. This reinforces the notion of "glutamine addiction" as a phenomenon common to the proliferative phenotype and to our knowledge is the first time a Glutaminase inhibitor has been successfully used to blunt lymphocyte proliferation.

5.0 CONCLUSION

Over fifty years ago Otto Warburg observed that cancer cells, unlike somatic cells, fermented glucose in the presence of available oxygen. Recently, this seminal discovery has led to the identification of a particular phenotype common to many proliferating cells, including mammalian lymphocytes. A key factor in this phenotype is the reliance on glutamine catabolism to provide anaplerotic carbon to the TCA cycle, and the generation of the reducing agent, NADPH.

This report has identified a specific Glutaminase enzyme, GAC, as up-regulated in proliferating CD4+ T lymphocytes. We also demonstrated the ability of two Glutaminase inhibitors, compound "968" and BPTES, to dampen this proliferation.

Unwanted lymphocyte proliferation is a hallmark of autoimmune diseases: a failure of an organism to recognize its own tissue and the subsequent immune response mounted against this tissue. Autoimmune diseases including Coeliac disease; Diabetes Mellitus (Type 1); Graves', Addison's & Sjøgren's disease; and Rheumatoid Arthritis (RA) are the second highest cause of chronic illness, and the top cause of morbidity in women, in the United States[80]. RA is particularly debilitating and affects 0.5-1.0% (700 million) of the world's population[81]. Treatments for these diseases are often very non-specific, broad inhibitors of cell proliferation which are associated with severe side-effects that greatly diminish recipients' quality of life. The identification of Glutaminase inhibitors that dampen unwanted lymphocyte proliferation represents a first step in the development of target-specific drugs that could potentially improve the quality of life for sufferers of autoimmune diseases throughout the world.

FUTURE PERSPECTIVES

The use of siRNA to target both *gls* and *gls2* genes, in addition to *myc,* would likely provide greater insight into how the genetic machinery regulates the expression of the Glutaminase isoforms in naïve and proliferating CD4+ lymphocytes. Similarly, analyses of the changes in the myc protein levels would also be of interest.

Sub-cellular localisation of the specific Glutaminase enzymes would confirm whether GAC's exclusivity found in certain cancer cells was applicable to T lymphocytes. It would also be interesting to know if intracellular phosphate concentrations change upon activation/proliferation, especially in the mitochondria.

Finally, using mass spectrometry to determine the fraction of glutamine-derived carbon that was converted to CO_2, lactate, alanine or citrate would provide a greater appreciation of glutamine's role in both naïve and proliferating CD4+ T lymphocytes.

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Appendix A – Additional Procedures

Slide-A-Lyser Dialysis Kit – removal of glutamine from cell medium

Modified directly from manufacturer's website.

A. Hydrate Membrane

1. Remove the cassette from its protective pouch. To prevent contamination, handle the cassette by the plastic frame only. Do not touch the membrane with ungloved hands. The 12-30mL Slide-A-Lyzer Cassette may be placed on end on a flat surface.

2. Hold the float chamber at the top of the unit and immerse cassette in dialysis buffer (PBS) for 2 minutes to hydrate membrane.

Note: Hydration increases membrane flexibility and allows it to adjust more readily to the positive pressure created as the sample is added and to the vacuum created when air is removed.

3. Remove cassette from buffer and gently tap the cassette edge on a paper towel to remove excess liquid. Do not blot the membrane.

B. Add Sample

1. Fill the syringe with the sample, leaving a small amount of air in the syringe. For large sample volumes, fill the syringe without the needle in place.

2. Orient the needle bevel sideways and penetrate the gasket through one of the syringe ports at a corner of the cassette. Slowly extend the needle into the cavity to a minimal extent and inject approximately half of the sample. For samples with high protein concentrations (e.g., 10mg/mL), fill the cassette slowly to avoid foaming.

3. Withdraw some air from the cassette by pulling up on the syringe piston and then inject remaining sample.

4. With the syringe needle inserted in the cassette cavity, withdraw remaining air from the cavity to compress the membrane windows so the sample contacts the greatest surface area. Use caution to prevent the needle from contacting the membrane. Minimal air left inside the cassette will not significantly affect dialysis efficiency.

5. Remove the syringe needle from the cassette while retaining air in the syringe. The gasket will reseal and the membrane cavity will contain minimal or no air. Mark the cassette corner with a permanent marker or record the number of the injected port.

C. Dialyze Sample

1. Float cassette in the dialysis solution of choice and stir gently to avoid creating a vortex that might pull the cassette down in contact with the stir bar.

Dialysis times for removal of glutamine:

1. Dialyse for 2 hours at room temperature.
2. Change dialysis buffer and dialyze for another 2 hours.
3. Change the dialysis buffer and dialyze overnight at 4°C.

D. Remove Sample

1. Fill syringe with approximately 15mL of air and penetrate gasket with the needle through a top, unused syringe guide port.

2. Slowly discharge air into cassette cavity to separate membranes, which prevents needle penetration of the membrane.

3. With the needle in place, turn the unit so that needle is on the bottom. Allow sample to collect near the port and withdraw sample into the syringe.

Pierce BCA Protein Kit – protein concentration

Modified directly from manufacturer's website.

Preparation of Standards and Working Reagent (required for both assay procedures)

A. Preparation of Diluted Albumin (BSA) Standards

Use Table 1 as a guide to prepare a set of protein standards. Dilute the contents of one Albumin Standard (BSA) ampule into several clean vials, preferably using the same diluent as the sample(s). Each 1mL ampule of 2mg/mL Albumin Standard is sufficient to prepare a set of diluted standards for either working range suggested in Table 1. There will be sufficient volume
for three replications of each diluted standard.

Table 1. *Preparation of Dilution Albumin (BSA) Standards*

Vial	Volume of Dileunt (uL)	Volume and Source of BCA (uL)	Final BSA Concentration (ug/mL)
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	Blank

B. Preparation of the BCA Working Reagent (WR)

1. Use the following formula to determine the total volume of WR required:

(# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required

Note: 2.0mL of the WR is required for each sample in the test-tube procedure, while only 200 µl of WR reagent is required for each sample in the microplate procedure.

2. Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). For the above example, combine 50mL of Reagent A with 1mL of Reagent B.

Note: When Reagent B is first added to Reagent A, turbidity is observed that quickly disappears upon mixing to yield a clear, green WR. Prepare sufficient volume of WR based on the number of samples to be assayed. The WR is stable for several days when stored in a closed container at room temperature (RT).

Microplate Procedure (Sample to WR ratio = 1:8)

1. Pipette 25µL of each standard or unknown sample replicate into a microplate well (working range = 20-2000µg/mL)

Note: If sample size is limited, 10µL of each unknown sample and standard can be used (sample to WR ratio = 1:20). However, the working range of the assay in this case will be limited to 125-2000µg/mL.

2. Add 200µL of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.

3. Cover plate and incubate at 37°C for 30 minutes.

4. Cool plate to RT. Measure the absorbance at or near 562nm on a plate reader.

Appendix B – List of solutions and reagents

NAME	SUPPLIER	CATALOG #
CD4+ isolation		
Phosphate Buffered Solution (PBS)	Sigma	D8537
RPMI1640	Sigma-Aldrich	R0883
Dynabeads CD4+ Isolation Kit	Invitrogen	11331D
Lysation		
NP40 Lysis buffer	Novex	FNN0021
PMSF Protease Inhibitor	Sigma	P7626
Protease Inhibitor Cocktail	Sigma	P8340
Ethanol	Merck	108543
Lgepal CA-360	Sigma	I3021
Pierce BCA Protein Assay		
Pierce BCA Protein Assay Kit	Thermo Scientific	23223
Western Blotting		
α-GAC antibody (Rb pAb)	Protein Tech	19958-1-AP
α-KGA antibody (Ms mAb)	Abcam	ab60709
α-LGA antibody (Rb pAB)	Abcam	ab91073
α-alpha-tubulin antibody (Rb mAb)	Sigma	081M4861
SDS (sodium dodecyl sulphate)	Biorad	161-0302
Glycine	VWR	101196X
NaCl	Merck	1.06404.1000
Methanol	Merck	MX0490
Tween 20	Sigma	101102360
TRIS	Calbiochem	648310
Precision Plus Dual Colour Standard	Biorad	161-0374
Criterion 12+2well 7.5% HCL-TRIS Gel	Biorad	345-0005
Criterion 18 well 7.5% HCL-TRIS Gel	Biorad	345-0006
PVDF Transfer Membrane	Millipore	IPVH00010
Super Signal West Pico Chemilumiescent Kit	Thermo Scientific	34080
Restore Western Blot Stripping buffer	Thermo Scientific	46430
Criterion Filter Paper	Biorad	1704085
CD4 stimulation		
Dynabeads CD3/CD28 Expansion Beads	Invitrogen	11131D
Dynabeads Mouse T-Activator CD3/CD28 Beads	Invtirogen	11456D
Interleukin 2 (IL-2)	PeproTech	200-02

[3H]-Thymidine CD4+ Stimulation Test		
[3H]-Thymidine	Nerliens	27X005MC
qPCR		
RNeasy Plus Mini Kit	Qiagen	74134
QuantiTect Reverse Transcription Kit	Qiagen	205311
β-mercaptoethanol	Sigma	129112
Rnase-free water	Qiagen	129112
TaqMan Gene Expression Kit	Applied Biosystems	4369016
TaqMan GAC Probe	Applied Biosystems	HS01022166_m1
TaqMan KGA Probe	Applied Biosystems	HS01014013_m1
TaqMan LGA Probe	Applied Biosystems	HS00998733_m1
TaqMan B2M Probe	Applied Biosystems	HS99999907_m1
Mass Spectrometry		
^{13}C-L-glutamine	Aldrich	607983
MS-grade Methanol	Merck	600-001-00
Glutamine removal		
Pierce Slide-A-Lyzer Dialysis Cassette 3.5K MWCO 30ml	Thermo Scientific	66130
Glutaminase inhibition		
Compound 968	SPECS	36107028
BPTES	Drug Discovery Lab AS	
Glutaminase *E.coli*	Sigma	G8880
L-GDH	Sigma	1001153002
NAD	Roche	10127965001
DMSO	Sigma	276855

Appendix C – Protein/RNA Concentrations

Nanodrop 2000c Spectrophotometer

Table x. Total protein concentrations in naïve and CD3/CD28-activated CD4+ T lymphocytes.

Donor	Sample	mg/ml	A280 absorbance	260/280 ab. ratio
1	Naive	19.31	19.31	0.56
	Activated	22.211	22.211	0.7
2	Naive	20.214	20.214	0.58
	Activated	22.599	22.599	0.7
3	Naive	16.502	16.502	0.73
	Activated	15.772	15.772	0.57
4	Naive	1.572	1.572	-0.51
	Activated	3.533	3.533	1.38
5	Naive	1.971	1.971	-0.13
	Activated	1.532	1.532	-0.63
6	Naive	2.681	2.681	0.92
	Activated	2.527	2.527	1.08

Table x. Total RNA concentrations in naïve and CD3/CD28-activated CD4+ T lymphocytes.

Donor	Sample	ng/ul	A260 absorbance	A280 absorbance	260/280 absorbance ratio
1	Naive	58.4	0.697	2.1	1.9
	Activated	266.8	3.206	2.08	2.12
2	Naive	256.5	3.084	2.08	2.12
	Activated	46.1	0.547	2.11	1.79
3	Naive	45.3	0.532	2.13	1.87
	Activated	237.4	2.87	2.07	2.13
4	Naive	271.8	3.287	2.07	2.11
	Activated	115.5	1.406	2.05	1.93

Pierce BCA Protein Assay

Table x. Total protein concentrations in naïve and CD3/CD28-activated CD4+ T lymphocytes.

Donor	Sample	560nm absorbance	ug/ml
7	Naive	0.300	774
	Activated	0.436	1225
8	Naive	0.233	550
	Activated	0.407	1130
9	Naive	0.405	1124
	Activated	0.268	667