ABSTRACT

Title of Document:	REGULATION OF GLUTAMINE UTILIZATION DURING T CELL ACTIVATION
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Activation of a naïve T cell is a highly energetic event, which requires an increase in metabolism. Upon stimulation, T cells increase in size, rapidly proliferate and differentiate, all of which lead to a high demand for energetic and biosynthetic precursors. Even though amino acids are the basic building blocks of protein biosynthesis, the role of amino acid metabolism in this process has not been well characterized. We have found that glutamine in particular is required for both proliferative as well as effector function. We have evidence that glutamine regulates ERK signaling and that ERK in turn may also regulate glutamine transport. These data indicate that glutamine may play a significant role in T cell signaling and that a better understanding of glutamine utilization in T cells may reveal novel targets for immunomodulatory and/or anti-leukemia therapy.

REGULATION OF GLUTAMINE UTILIZATION DURING T CELL
ACTIVATION

By

Erikka Carr

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
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Professor Kenneth Frauwirth, Chair
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Dedication

To all my friends and family especially my mother, Jennelle Vanish.

Acknowledgements

It has been a long three years but I owe thanks to many for getting me to this point. I would like to thank my advisor, Ken Frauwirth for all of his guidance and support. Most of all I thank him for being so patient with me and always being available to answer questions. I would like to thank the other members of my thesis committee, Eric Baerecke and Wenxia Song for their suggestions on how to further my project. I would also like to thank Brooke Humphrey for all of his help with the real time PCR project. He helped me tremendously with learning the technique and how to interpret the data as well as giving full assess to his equipment. I am especially thankful for all of the members of the Frauwirth lab for making my time here memorable. A special thanks to Mathangi Srinivasan for her guidance as well as being a great friend. I could always count on her for help with my experiments or to take a venting break when I thought I couldn't take it anymore. To all the other lab members: Aimee, Paul, Anahit, Heather, Nikki and Susan, thanks for being there for me. I would also like to thank Glendon Wu and Vinny Gopaul for all of their hard work on assisting me on my project. They were both wonderful students to work with and I am forever indebted them. There are many people in the department who have contributed to my success here and I am grateful for them. I am eternally grateful for the support from Jessica Miller and the Song lab and giving helpful suggestions for my project. They were there for scholarly advice as well as being good friends. Finally, I would like to thank my family and friends for always encouraging me to strive for the best in all that I do. I would especially like to thank my mother for always being there to listen even if she didn't understand what I did in the lab everyday. To all who have contributed to getting me this far, I appreciate it and I will miss you all!

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Introduction

Amino acids play a pivotal role in cell growth and proliferation. Although their significance in protein biosynthesis is well documented, their role in regulating signal transduction is much less characterized. Amino acids act as nutritional signals that alter gene expression by modulating mRNA translation. By constantly monitoring amino acid availability, cells can regulate signaling pathways leading to translation initiation. Cellular response can either result in global changes in mRNA translation or changes in a subset of mRNAs (Kimball and Jackson 2004). During antigen engagement with the T cell receptor, there are many signaling processes that are required to regulate whether the cell should be activated, when to grow, and when these processes should cease. Therefore, amino acids are critical for proper T cell function. Here I propose that amino acids are key regulators of T cell activation and that glutamine in particular plays an important role in transport of other amino acids, amino acid sensing, and in signaling pathways that regulate T cell activation. This thesis examined glutamine is not merely an energy source but acts as a signaling regulator which is required to activate signaling pathways required for proper T cell activation.

T cell Activation

T lymphocytes are key mediators of the adaptive immune response. These cells are activated when antigen is presented to them on the MHC molecules of antigen presenting cells. However, the activation of T cells also requires co-stimulation. Co-stimulation involves T cell recognition of accessory molecules called B7 molecules present on the

antigen presenting cells (APCs). Co-stimulatory molecules act to work synergistically with the antigen to activate the T cell. In fact, co-stimulation by CD28 may play a role in T cell metabolism (Frauwirth 2004). The T cell receptor/CD3 complex receives the signal which initiates a signaling cascade leading to activation of the T cell (Figure 1). The TCR is phosphorylated at its zeta chain at immunoreceptor tyrosine-based activation motifs (ITAMs) by the protein tyrosine kinases LCK and FYN. ZAP70 is recruited to the active zeta chain where it phosphorylates other signaling molecules that promote numerous intracellular signal transduction pathways. Some of these pathways include RAS activation, calcium mobilization, polarization of the actin cytoskeleton and protein kinase C activation. Transcription factors NF kappa B and NFAT promote the transcription of genes which give rise to cytokine secretion and T cell proliferation. Activated T cells in turn recruit other important immune cells leading to a productive adaptive immune response (Baniyash 2004).

Markers of Activation

When the T cell receptor is engaged, it stimulates T cells to express a series of proteins which are necessary for a productive immune response. Because expression of these proteins is indicative of T cell activation, they are commonly used as activation markers. Some of the most common activation markers are CD25, CD44, CD69 and 4F2. CD25 is the alpha subunit of the IL2 receptor. Shortly after TCR engagement, CD25 is expressed on the cell surface allowing secreted IL2 to bind to it and further signal the T cell to proliferate (Robb 1981). CD69 is known to be rapidly induced during lymphocyte activation. It is not expressed at the cell surface in resting cells but is actively expressed
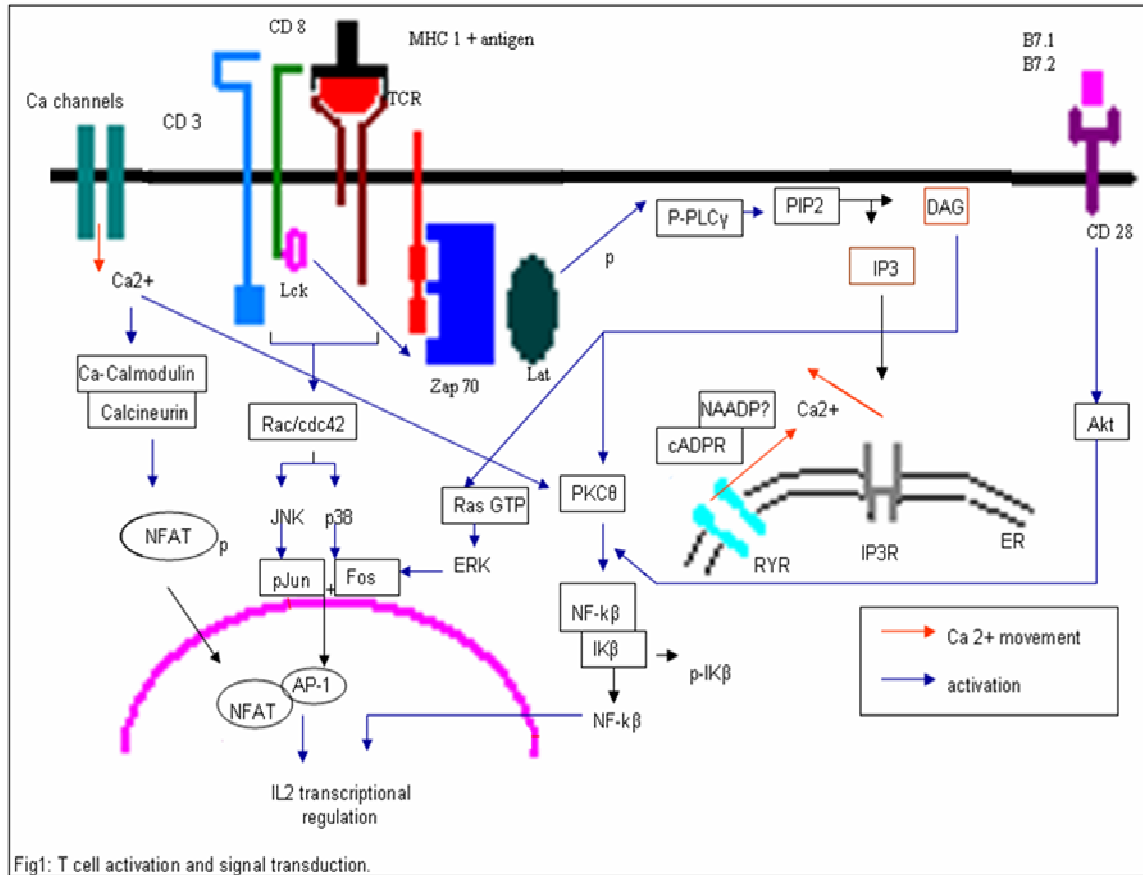
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Fig1: T cell activation and signal transduction.

Figure 1. A *Representation of T cell Activation.* When the T cell receptor/CD3 complex is engaged with antigen, it receives the signal which initiates a signaling cascade leading to activation of the T cell. The TCR is phosphorylated at its zeta chain by the protein tyrosine kinases LCK and FYN at their immunoreceptor tyrosine-based activation motifs (ITAMs). ZAP70 is recruited to the active zeta chain where it phosphorylates other signaling molecules that promote numerous intracellular signal transduction pathways. Some of these pathways include RAS activation, calcium mobilization, polarization of the actin cytoskeleton and protein kinase C activation. Transcription factors NF kappa B and NFAT promote the transcription of genes which give rise to cytokine secretion and T cell proliferation. Activated T cells in turn recruit other important immune cells leading to a productive adaptive immune response.

on the surface of activated cells within 2 hours of stimulation. Upon, stimulation, CD69 induces IL2 and IFNγ production as well as inducing Ca+ influx (Hamann 1993). CD44 expression is known to be upregulated in activated lymphocytes in order to facilitate movement throughout the extracellular matrix through interactions with hyaluronic acid and fibronectin (Hogg 1993). 4F2 is a heterodimeric glycoprotein that is often associated with early T cell activation events involving T cell adhesion (Rintoul 2002). It is also an amino acid transporter component that is thought to be important for membrane targeting (Verrey 2003).

Amino Acids as Signaling Regulators

T cell activation is a highly energetic process which requires an increase in metabolism. In order for cells to maintain a level of metabolic homeostasis, increased uptake and metabolism of nutrients must occur. Once activated, T cells undergo rapid growth and proliferation, increasing significantly in size from its resting state and secreting cytokines necessary for eliciting an immune response. Although amino acids are the basic building blocks of protein synthesis, the role that amino acid utilization plays in the conversion from a resting to an activated T cell is poorly understood. Several genes associated with amino acid transport and amino acid biosynthesis are induced under starvation conditions in various cell types including T cells (Fafournoux 2000, Aulak 1999, Peng 2002). Taken together, these data indicate that amino acids may play a crucial role in proper T cell function.

There is evidence suggesting that amino acids and their biosynthetic precursors are not

merely used for energy but could also be involved in signal transduction (Christie 2001). Signaling pathways such as GCN2 and mTOR show that cells have the ability to sense the concentration of amino acids and alter gene transcription and protein translation based on amino acid availability (Kimball and Jefferson 2004). Besides being sensed, the presence or absence of certain amino acids can affect signaling pathways that may not be involved in protein biosynthesis at all such as MAPK signaling (Franchi-Gazzola 1999). Amino acids are also known to affect T cell activation at the level of the TCR. Arginine is an essential amino acid for T cells, as depletion of this amino acid in T cells causes down-regulation of the TCR zeta chain expression upon stimulation. In primary mouse T cells, there is a defect in recycling of the TCR zeta chain, while the leukemic Jurkat T cells have heightened zeta chain degradation when starved of arginine (Zea 2004; Taheri 2001; Rodriguez 2002).

The goal of this thesis is to examine the role of amino acid availability in regulating T cell activation. In particular, we will characterize the importance of glutamine during T cell activation. We will investigate how it is sensed, the downstream effects of glutamine starvation, and the mechanisms used to regulate glutamine transport.

Chapter 1: Activation effects of glutamine depletion

Importance of Glutamine in T cells

Glutamine is the most abundant free amino acid in blood and is a primary energy source in rapidly dividing cells, including activated T cells, because of its ability to enter the Krebs Cycle (Mates 2006, Ham and McKeehan 1979). In fact, lymphocytes are thought to use glutamine as much as or more than glucose (Newsholme 1985). Glutamine plays an important role in many cellular processes, including cell growth, proliferation, and protein synthesis and degradation (Gu 2001). Low levels of glutamine have been associated with impairment of T cell function, such as proliferation and secretion of IL-2 and IFNγ (Newsholme 2001). Glutamine depletion has also been shown to regulate expression of the IL-2 receptor (Yaqoob and Calder 1997). Glutamine is also thought to play an important role in preventing apoptosis, as glutamine supplementation significantly decreases apoptosis and caspase 3 and caspase 8 activity in activated T cells (Mates et al 2006).

Most of the glutamine taken up by the cell is converted into glutamate and aspartate (Newsholme 2001). Being a precursor for glutamate, glutamine is a good source for glutathione production and purine synthesis (Doepel 2006). Studies have shown that glutamine supplementation protects T cells from ROS mediated oxidative stress by increasing glutathione production. Conversely, when T cells are starved of this amino acid, there is a rise in the concentration of ROS, which leads to activation of several

signaling cascades such as calcium signaling and MAPK signaling (Mates 2002).

In this first chapter, we performed a comprehensive study in which we established the specific amino acids that were essential for T cell activation using a mouse model. We then continued by further characterizing the importance of glutamine availability during T cell activation in an effort to establish glutamine not only as a source of energy but also as a major signaling regulator.

Materials and Methods

Mice

C57BL/6J mice were obtained from Jackson laboratories. Mice carrying a transgene for 2C TCR have been described previously (Sha et al., Nature 1988).

Reagents

Amino Acid Free RPMI was made in the lab following the formula from MP Biomedicals (Irvine,CA). Amino acids were added individually as needed. RPMI was supplemented with 10% fetal bovine serum, Pen/Strep, 55 µM β-mercaptoethanol and 10 mM HEPES buffer.

T cell Purification

T cells were purified from the spleen of either wild-type B6 mice or from 2C TCR transgenic mice (Sha et al., Nature 1988) using the SpinSep Purification Kit (Stem Cell Technologies, Vancouver, BC) following the manufacturer's instructions.

Generation of bead-linked antibodies

Anti-CD3/anti-CD28 antibodies were linked to tosyl-activated magnetic beads following

a modified version of the manufacturer's protocol (Dynal). 4 x 10^8 magnetic beads were washed twice with Buffer B (0.1M borate buffer, pH 9.5) for 5 minutes, then incubated with 75 µg/ml each of anti-CD3 and anti-CD28 antibody resuspended in Buffer B overnight at 37ºC with constant rotation. Beads were removed from the antibody mixture with a magnet then washed twice in cold Buffer D (1X PBS with 0.1% BSA, pH 7.4) for 5 minutes each at 4ºC. Beads were washed in Buffer E (0.2 M Tris with 0.1% BSA, pH 8.5) overnight at room temperature then once in Buffer D for 5 minutes at 4ºC. Antibody-conjugated beads were resuspended in Buffer D and used at 3 beads per T cell.

Proliferation Assay

Amino acids were systematically depleted from RPMI and T cell activation was assessed by measuring proliferation. We used two different approaches for assessing T cells proliferation. One method required using purified T cells from mice transgenic for the 2C TCR receptor. T cells were plated in 96-well plates at 5 x 10^4 cells per well and stimulated with 1:5 serial dilutions of peptide specific for the 2C receptor with the starting concentration being 200 nM. Splenocytes from C57BL/6J mice were used as antigen presenting cells at a concentration of 2 x 10^5 cells per well. Our second approach used unfractionated splenocytes of B6 mice plated at 2.5 x 10^5 cells per well. T cells were stimulated with 10 µg/ml anti-CD3 antibody as the starting concentration which was serially diluted 1:10. Proliferation was measured after 72 hours by uptake of [^{3}H] thymidine. T cells were incubated with 1µCi/well of [^{3}H] thymidine for 6-8 hours and transferred to glass fiber filters using a 96-well harvester (Tomtec). Uptake was determined by liquid scintillation using a Microbeta Trilux scintillation counter (Wallac, Turku, Finland).

ELISA

Production of IL2 and IFNγ were assessed by sandwich ELISA using supernatants of stimulated cells cultured for 36 hours. Primary and biotinylated secondary antibodies against each cytokine as well as cytokine standards were used at concentrations recommended by the manufacturer (eBioscience, San Diego,CA). Alkaline phosphatase-conjugated avidin was used at a 1/3000 dilution (Jackson Immuno-Research Laboratories) and the colorimetric alkaline phosphatase substrate that was purchased from Sigma-Aldrich was used at a concentration of 1 mg/ml in 10% diethanolamine buffer. Levels of secreted IL2 and IFNγ were assessed using the SoftMax Pro 4.6 software.

Activation Marker Expression and Viability Testing

Expression of activation markers was measured via flow cytometry. Cells were allowed to rest or were stimulated with beads conjugated with anti-CD3 and anti-CD28 antibodies for 20 hours in the presence or absence of glutamine. Expression was measured using PE-linked antibodies specific for CD25, CD44, CD69 or 4F2 (all purchased from eBioscience) for each condition. All cell samples were co-stained with FITC-linked anti-Thy1.2 (eBioscience) to gate on T cells. To test for viability, cells were stained with propidium iodide (Molecular Probes) after 24 hours of stimulation.

Results

Glutamine is required for T cell Activation

Although several studies have shown that specific amino acids are necessary for T cell activation, there have been few comprehensive studies using primary T cells. To address

this, primary T cells were stimulated in media individually lacking each amino acid. It was found that many amino acids are required for T cell activation. As expected, amino acids that cannot be synthesized in the body and are therefore required to be taken up in the diet, such as leucine, methionine and lysine, were required for T cell activation. However, T cell activation was greatly disrupted by lack of some amino acids that can be synthesized from other amino acids and are traditionally considered nonessential amino acids. These amino acids included glutamine, arginine, and asparagine (Figure 2 and 3). In fact, glutamine depletion had the greatest effect on T cell activation. Further examination of the effects of glutamine depletion show that proliferation was reduced more than 90%, while depletion of its cationic counterpart, glutamate did not affect proliferation. Glutamine depleted T cells were also deficient in production of IL-2 and IFNγ, where IFNγ production was undetectable in the absence of glutamine (Figure 3). To ensure that glutamine depletion was not negatively affecting proliferation by increasing cell death, we measured cell viability after 24 hours by propidium iodide exclusion. Glutamine starved cells were viable and were not undergoing cell death after 24 hours in culture (Figure 4). To determine the minimum amount of glutamine required for normal T cell proliferation, a titration assay was performed using ten-fold dilutions of glutamine. T cells were able to proliferate with as little as 10% of the typical amount of glutamine used in culture, which is approximately the normal serum concentration. However, when glutamine concentrations were reduced, T cell proliferation required a higher concentration of antigen stimulation, leading to a shift in the dose response, suggesting that T cells may be sensing extracellular glutamine concentrations (Figure 5). In contrast, arginine limitation inhibited the cells' ability to proliferate, and increasing
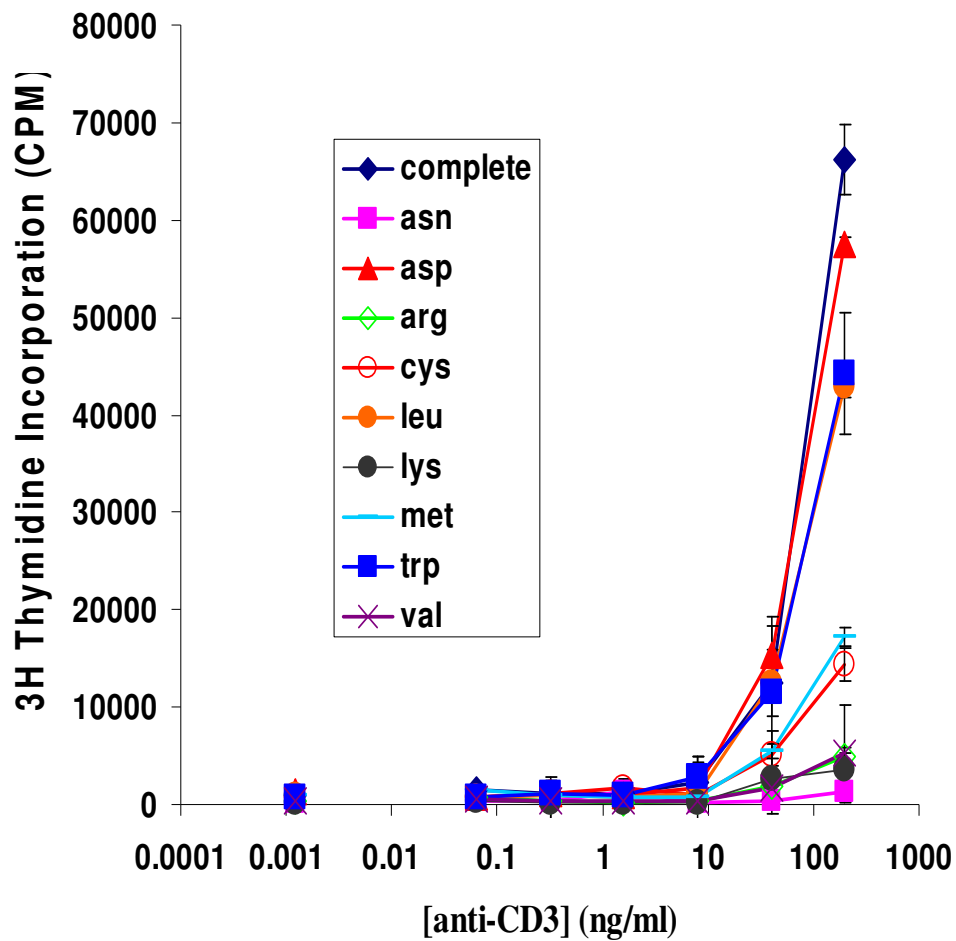
Figure 2. *T cells Require Specific Amino Acids for Proliferation.* Splenic T cells wereUnfractionated splenocytes were purified (are these really purified T cells, or unfractionated splenocytes?) and cultured in RPMI depleted of individual amino acids. T cells were stimulated with anti-CD3 antibody and proliferation was measured after 72 hours using [^{3}H] thymidine incorporation. Error bars represent ± standard error of samples analyzed in triplicate.
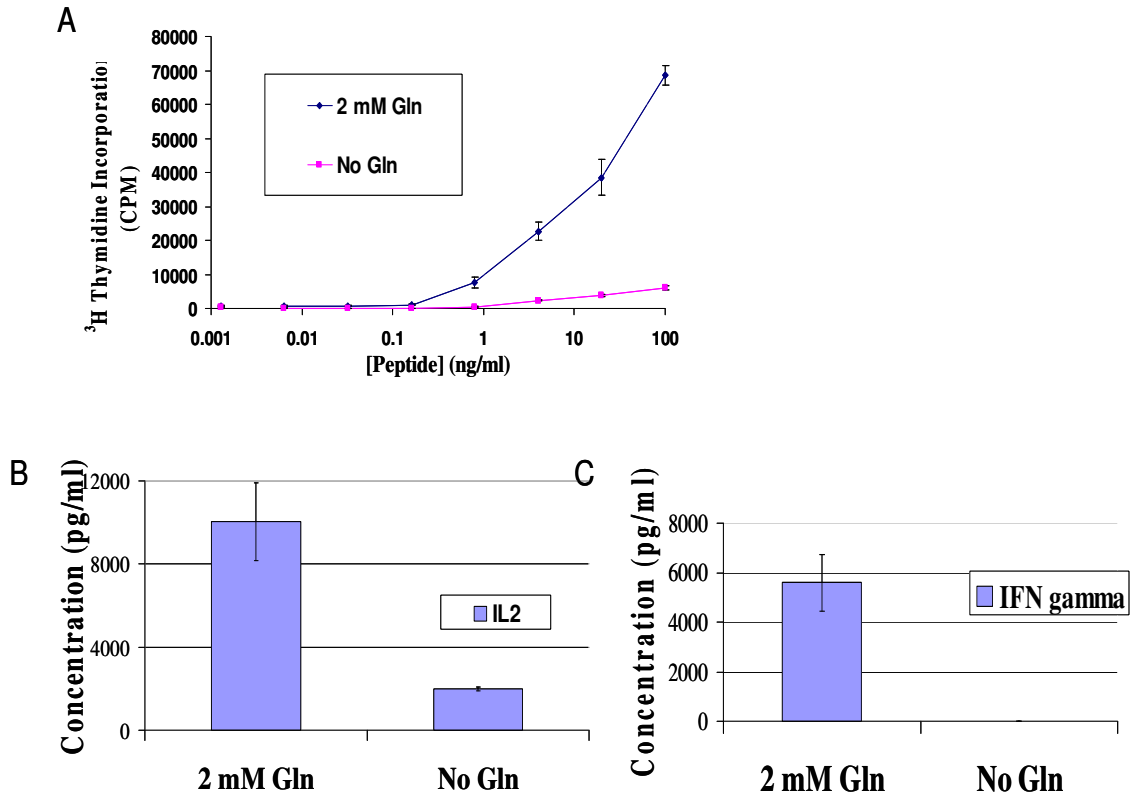
Figure 3. *Glutamine is required for T cell activation.* Purified T cells were grown for 72 hours at 37˚C in the absence of glutamine and proliferation was measured by uptake of [3H] thymidine over the last 8 hours of incubation (A). Secretion of IL2 (B) and IFNγ (C) was measured using sandwich ELISA after 72 hours. Error bars represent ± standard error of samples analyzed in triplicate.
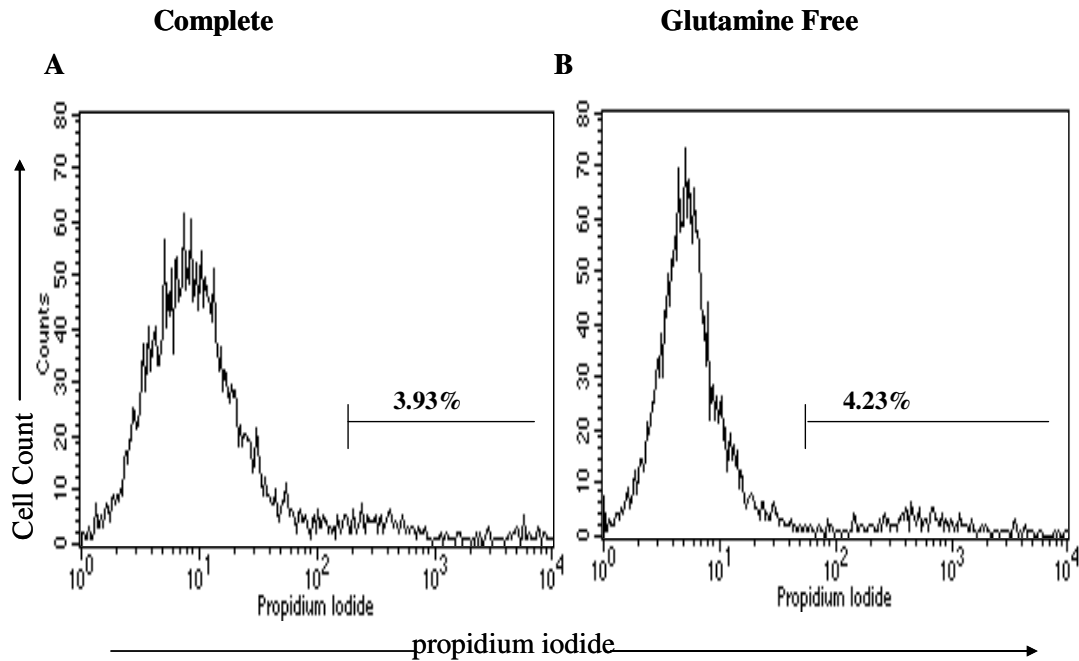
Figure 4. *Glutamine starved cells are viable after 24 hours.* Purified T cells were stimulated for 24 hours with anti-CD3 antibody in RPMI containing 2 mM glutamine or under glutamine-free conditions. T cells were then co-stained with Thy1.2 FITC and propidium iodide and analyzed by flow cytometry. Viability was measured in T cells cultured in RPMI containing glutamine (A) and glutamine-free RPMI (B) by propidium iodide exclusion of Thy1.2 positive T cells. Percentages represent propidium iodide-positive Thy1.2 positive T cells.
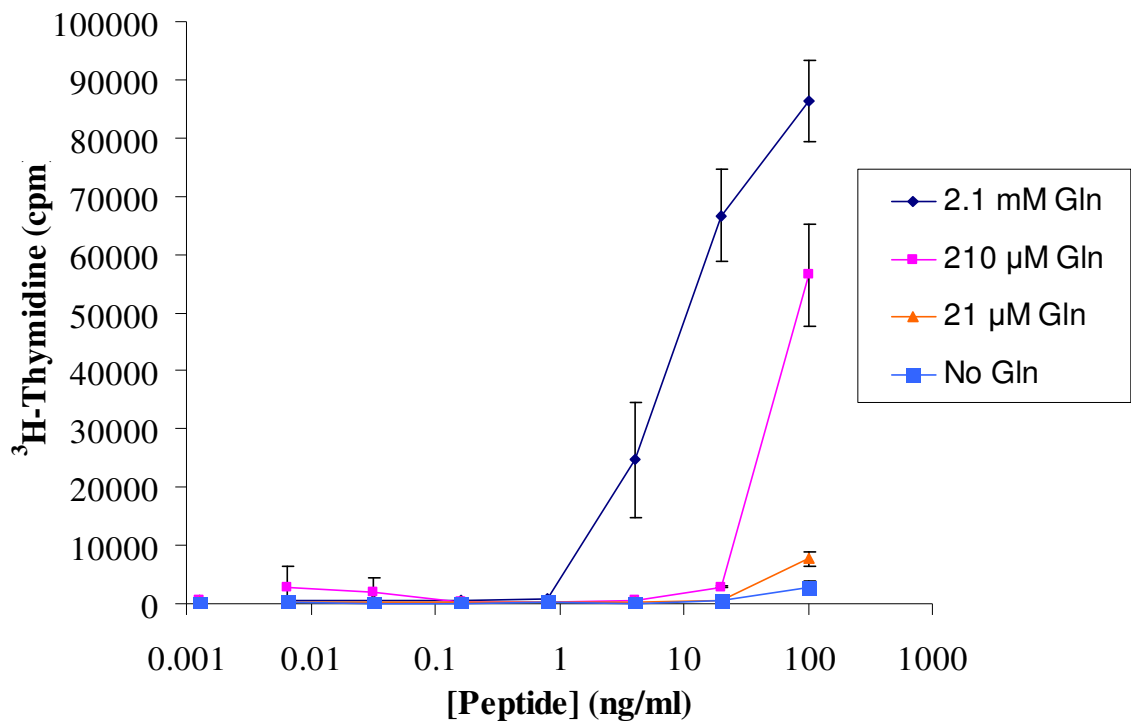
Figure 5. *Glutamine is sensed in primary T cells*. Purified T cells were cultured for 72 hours at 37°C in the presence of serially diluted concentration of glutamine. Proliferation was measured by [^{3}H] thymidine incorporation. Error bars represent ± standard error of samples analyzed in triplicate.
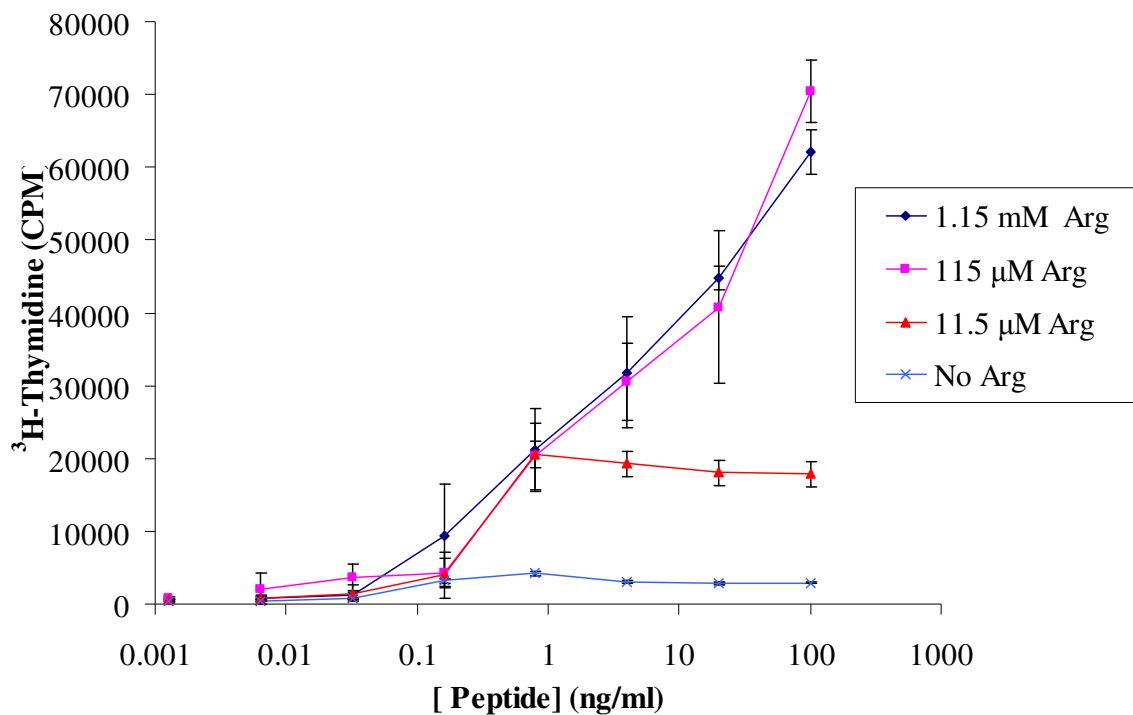
Figure 6. *Arginine is not sensed in primary T cells*. Purified T cells were cultured for 72 hours at 37°C in the presence of serially diluted concentrations of arginine. Proliferation was measured by [^{3}H] thymidine incorporation. Error bars represent ± standard error of samples analyzed in triplicate.

peptide stimulation could not compensate for the lack of arginine (Figure 6). Taken together, these findings would suggest that glutamine reduction may be limiting cell signaling while reduction in arginine may be limiting cellular metabolism (discussed in Conclusion).

Most of the glutamine taken up by cells is converted into glutamate and aspartate (Newsholme 2001). In rat lymphocytes, glutamine utilization is high but oxidation is fairly low. Most of the glutamine that is taken up is then converted solely into glutamate (Newsholme 2001). These cells may therefore require glutamine merely as a source of glutamate. If this were true, T cells may be sensing intracellular levels of glutamate rather than glutamine. The next aim was to determine whether glutamine itself was required or whether its primary metabolite, glutamate was the required amino acid. Glutamate was added to glutamine depleted media at 2 mM, the normal concentration of glutamine in culture, and proliferation was measured. Increasing the concentration of glutamate was unable to rescue the proliferative or effector defect in glutamine starved T cells (Figure 7). This suggested that either glutamate was unable to be taken up fast enough or that glutamine is the sensed molecule required for proper function. We hypothesized that both circumstances are likely because glutamate transporters are usually at low density compared to other amino acid transporters (Newsholme 2003).

If glutamine was required solely as an energy source to be converted to glutamate, then any glutamine/glutamate precursor should be able to substitute for the loss of glutamine in the system. To test this, proliferation was examined under glutamine-free conditions

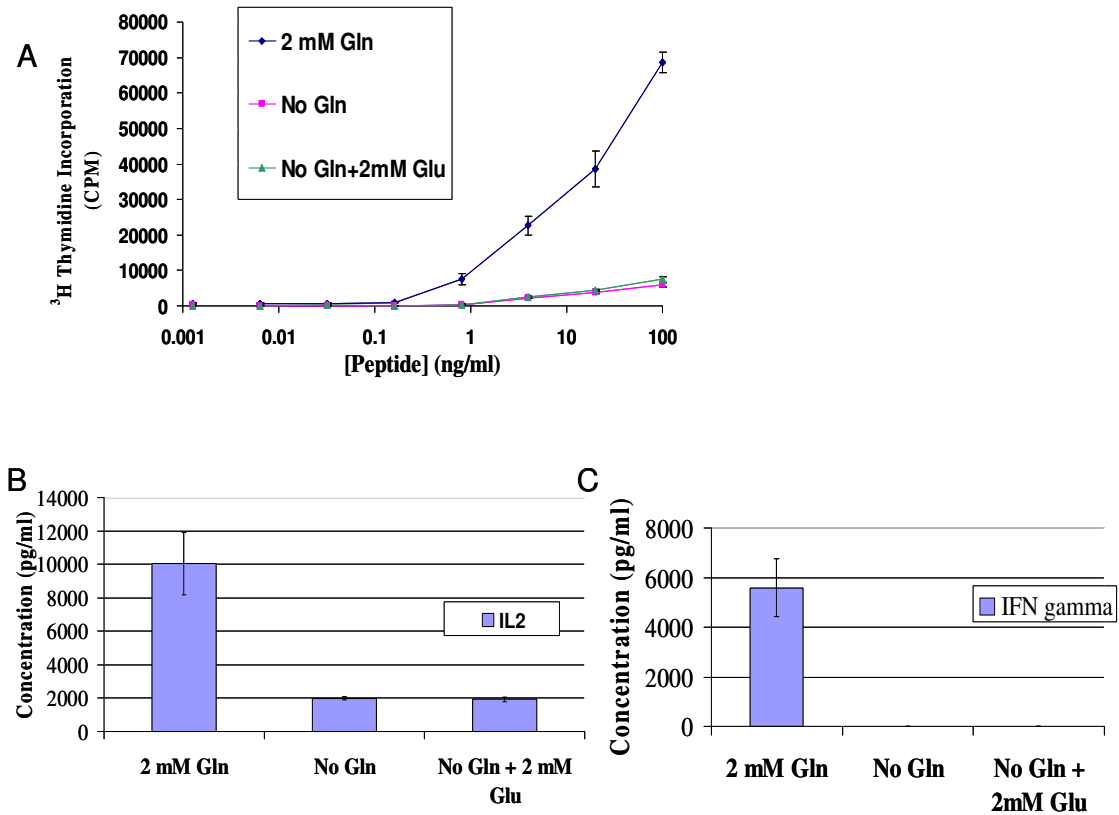Figure 7. *Glutamate supplementation does not compensate for glutamine deficiency.* Splenic T cells were purified and cultured in the presence of 2 mM glutamine or in glutamine-free RPMI supplemented with 2 mM glutamate. T cells were stimulated with 2C peptide and proliferation was measured after 72 hours using [3H] thymidine incorporation. Error bars represent ± standard error of samples analyzed in triplicate.

but supplemented with 2 mM concentrations of glutamine precursors. Proline and asparagine were chosen because they were both capable of being converted into glutamate/glutamine but unlike glutamate, could be transported by a variety of transporters which are expressed in T cells (Peng 2002). However, supplementation of glutamine-free RPMI with either of these precursors was insufficient in substituting for glutamine (Figure 8). This implies that either T cells are less efficient at transporting in glutamine precursors or that glutamine itself is required. The former is less likely because proline and asparagine can be transported through transporters that are highly expressed in T cells (Peng 2002).

Glutamine Acts Downstream of Early T cell Activation

From our data thus far, we observed that T cell activation may be regulated by glutamine sensing. We have measured glutamine's effects on late T cell activation markers such as proliferation and cytokine production. Our next step was to ask if glutamine may be acting much earlier in T cell activation. To address this, we examined key early activation markers when cells were glutamine depleted. When T cells were starved of glutamine for 20 hours, expression of CD44, CD69 and 4F2 **was** nearly identical to T cells grown under normal conditions. However, there was a slight decrease in surface expression of CD25 in glutamine starved cells (Figure 9). This suggests that glutamine may play a limited role in early T cell activation events.

Conclusion

We have confirmed that the presence of glutamine in the T cell environment is critical for
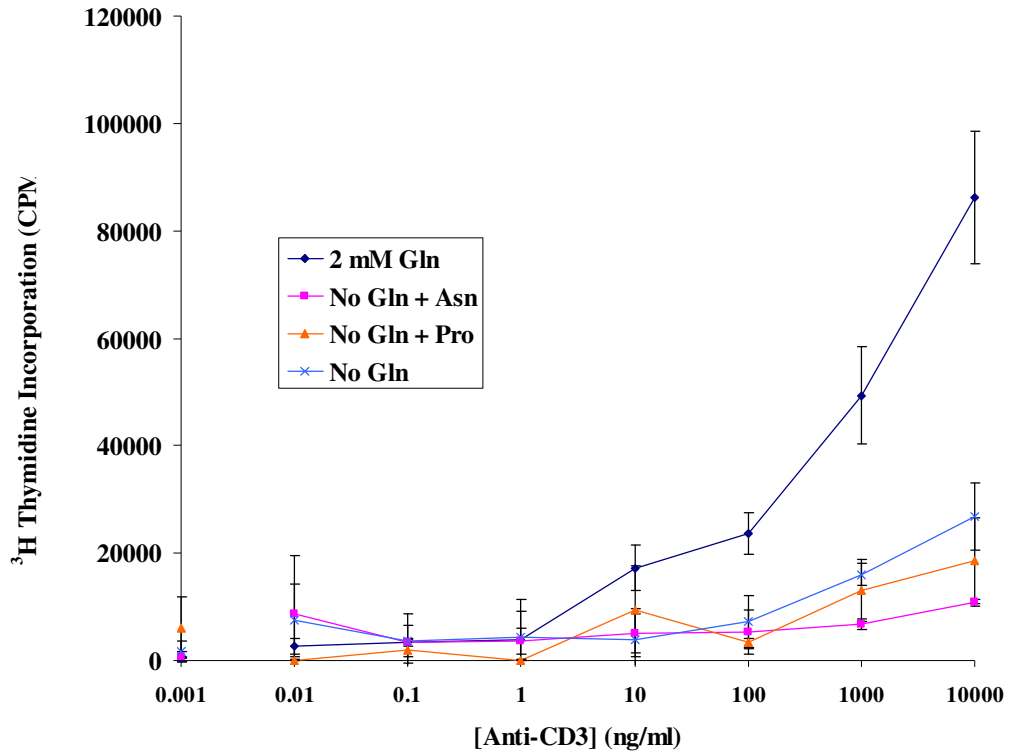
Figure 8. *Glutamine precursors do not compensate for the absence of glutamine*. Splenic T cells were purified and cultured in the presence of 2 mM glutamine or in glutamine-free RPMI supplemented with 2 mM of asparagine or proline. T cells were stimulated with anti-CD3 antibody and proliferation was measured after 72 hours using [^{3}H] thymidine incorporation. Error bars represent ± standard error of samples analyzed in triplicate.
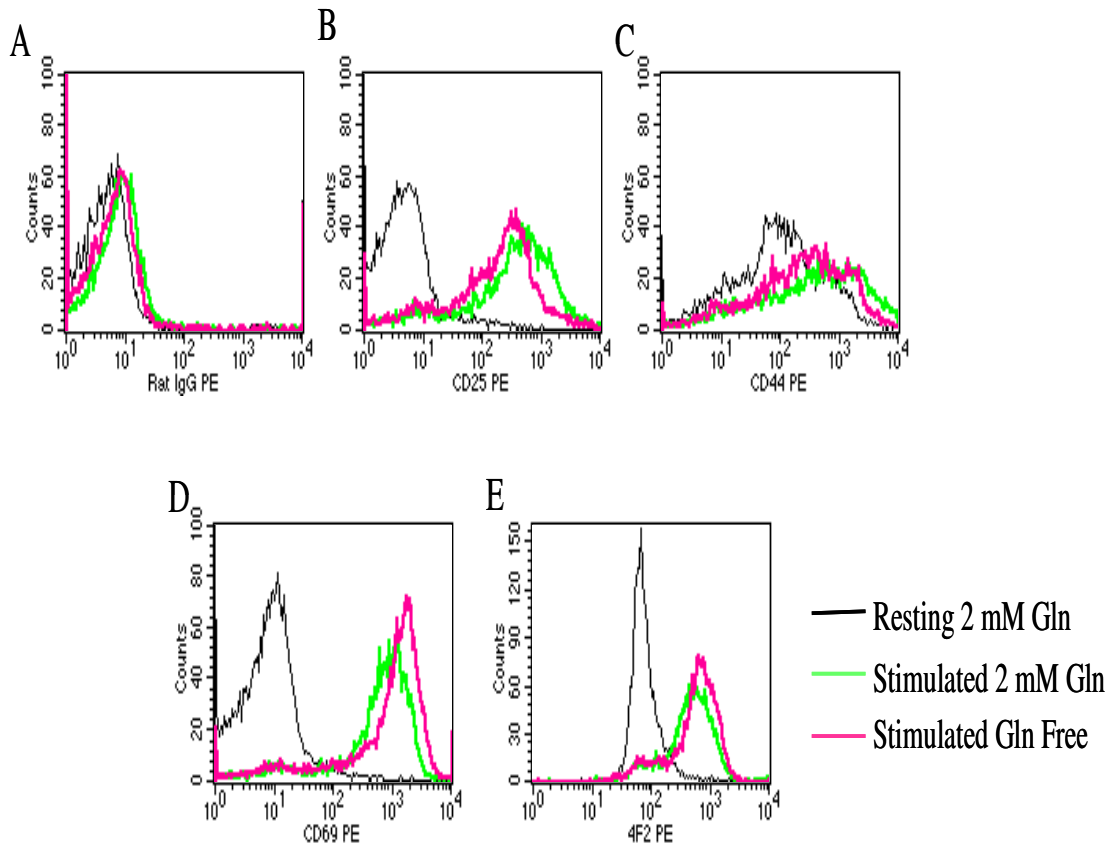
Figure 9. G*lutamine shows limited regulation of early activation markers*. Splenic T cells were purified and stimulated for 20 hours using anti-CD3/ anti-CD28 conjugated beads in the presence or absence of glutamine. T cells were stained with PE-linked rat IgG (A), CD25 (B), CD44 (C), CD69 (D) and 4F2 (E) then analyzed by flow cytometry. T cells left in culture overnight without stimulation (black) had low level expression of all activation markers compared to those stimulated in the presence of glutamine (green) or under glutamine-free conditions (pink).

T cell activation. These data have provided strong implications that glutamine may be acting as a signaling molecule for the cell to determine whether environmental conditions are appropriate for activation. We have developed a working model to demonstrate what we perceive to be the way in which amino acids contribute to T cell proliferation (Figure 10). As illustrated in Figure 10, under normal growth conditions, cells require amino acids for fuel as well as stimulation in order to proliferate. If an amino acid that acts primarily as a fuel source is significantly reduced, the cell will still respond to its stimulation signal but because it does not have enough fuel, it can only proliferate until that source is depleted. However, if an amino acid is acting as a signaling molecule, depletion of that amino acid will delay cellular response and would require more signals from another source, in this case through stimulation via the T cell receptor. Because T cells can overcome glutamine reduction with more antigen stimulation, we hypothesize that glutamine regulates signaling pathway(s). After examining levels of early activation markers, we can conclude that in response to glutamine depletion, T cells differentially express these activation markers. While the expression profile of glutamine starved T cells were nearly identical to T cells supplied with glutamine, CD25 expression was downregulated. Taken together, our findings suggest that glutamine may play a more important role downstream of early T cell activation signals. From this data, it is necessary to further characterize the importance of glutamine utilization by unveiling the sensing mechanism used by T cells. To address this, we have examined several well characterized amino acid sensors as well as some potentially novel sensors to determine which regulate T cell response during glutamine deprivation.
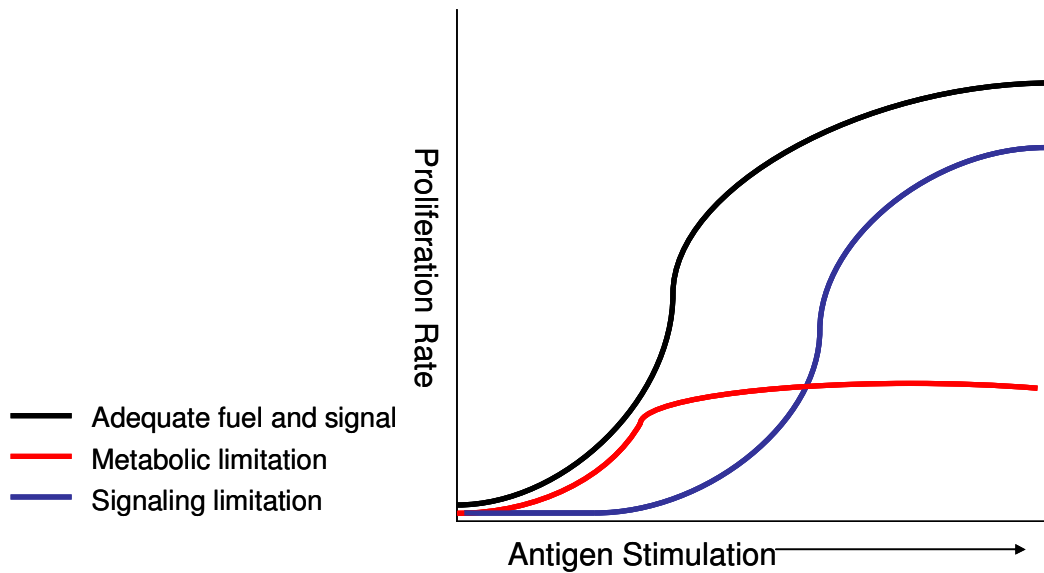
Fig 10. *Hypothetical Representation of Amino Acid Regulation of T cell Activation.* Under normal growth conditions, cells require amino acids for metabolism as well as stimulation in order to proliferate (black line). If an amino acid that acts primarily as a metabolic substrate is significantly reduced, the cell will still respond to its stimulation signal but because it does not have enough of the substrate, it can only proliferate until that source is depleted (red line). However, if an amino acid is acting as a signaling molecule, depletion of that amino acid will delay cellular response and would require more signals from another source, in this case through stimulation via the T cell receptor (blue line). From our data, this suggests that T cells grown in a lower concentration of glutamine may require more antigen stimulation in order to overcome the glutamine deficiency.

Chapter 2: Identification of Signaling Pathways that Sense Glutamine

Introduction

Like many cells in the body, in order to have homeostatic regulation of glutamine metabolism, T cells must have a tightly regulated sensing mechanism. Cells must adopt a mechanism which can monitor nutrient concentrations and rapidly react in response to these environmental cues by coordinating the enzymatic reactions necessary for nutrient metabolism. Several signaling molecules have been characterized as nutrient sensors in a variety of cell types. The signaling pathways which have been implicated in amino acid sensing include mammalian target of rapamycin (mTOR), the general control non-derepressible kinase 2 (GCN2) and mitogen activated protein kinase (MAPK) pathways (Kimball and Jefferson 2004, Franchi-Gazzola 1999).

Amino acid sensing through mTOR

One of the best characterized amino acid sensors is mTOR, which is a key mediator of cell growth, proliferation and metabolism. mTOR associates with two regulatory proteins, raptor and GβL, and is dependent on both for activation. mTOR regulates mRNA transcription and protein translation in response to amino acid availability and other essential nutrients in the environment. It surveys amino acid availability and acts as a master switch that determines whether a cell will grow and proliferate (Inoki 2005). mTOR is known to sense branched amino acids, which include leucine, isoleucine, and valine; however, the mechanism by which mTOR does this is unknown. Some studies suggest amino acids target the stability of the mTOR/raptor/GβL complex (Kim 2003). Well characterized downstream targets of mTOR include p70S6K and 4EBP1, both of

which control protein translation. Phosphorylation of ribosomal protein S6 by S6K leads to a heightened capacity of cells to translate protein. On the other hand, 4EBP1 acts as a translational inhibitor by binding to and inhibiting the translation initiator eIF4E. When mTOR signaling is activated, it activates S6K while suppressing 4EBP1 activity (Inoki 2005).

The GCN2 Amino Acid Sensing Pathway

There are many signaling pathways which modulate protein translation. GCN2 (general control non-derepressive kinase 2) belongs to a family of kinases that phosphorylate eIF2α, a key initiation factor for translation. When phosphorylated, eIF2α is converted into an inhibitor, blocking translation initiation. Amino acid availability plays a pivotal role in eIF2α phosporylation. Depletion of any one essential amino acid can lead to increased eIF2α phosphorylation and subsequent decrease in eIF2B activation in liver and skeletal muscle (Anthony 2001), as well as cells in culture (Kimball 1998).

GCN2 is of particular interest because it acts as a sensor of amino acid availability. This protein kinase regulates translation by monitoring levels of uncharged tRNA. GCN2 resembles a typical eukaryotic protein kinase except it contains a domain that resembles a histidyl tRNA synthetase. This domain has a higher binding affinity to uncharged tRNA than charged tRNA. When amino acid availability is low, there is less coupling of tRNA to free amino acids, leading to an increase in uncharged tRNA. Activation of GCN2 by uncharged tRNA turns translation off (Dever 2005). Although this pathway leads to a global inhibition of protein translation, a specific subset of mRNAs responsible for

controlling stress conditions is upregulated. These gene products become useful in monitoring signaling activity. For example, since there is not a good assay to measure GCN2 activity directly, downstream targets such as CHOP (C/EBP homology protein) are commonly used markers for GCN2 activity. CHOP, also known as Gadd153, is a transcription factor which is markedly induced by a variety of cellular stresses, such as nutrient deprivation, endoplasmic reticulum stress, and growth arrest (Schmitt-Ney 2000). Amino acid starvation in many cell types induces CHOP expression. For example, depletion of the essential amino acid tryptophan in murine T cells up-regulates CHOP expression (Munn 2005). Studies have also shown that mouse embryo fibroblasts starved of leucine rapidly induce CHOP (Harding 2000). CHOP can also be activated by GCN2-independent mechanisms. For example, other stress activated pathways such as MAPK can affect CHOP expression (Hai 1999, Wang 1996, Fan 2002). Although CHOP expression is often used as an indicator of GCN2 activity, CHOP is activated by many different stresses, and is several substrates downstream of GCN2. GCN2 activity can also be measured by other GCN2 targets, such as eIF2α phosphorylation and ATF4 expression, both of which are upstream of CHOP.

MAP Kinases as Potential Amino Acid Sensors

Traditionally, mTOR and GCN2 are most associated with sensing amino acids; however other pathways may also play a critical role in amino acid sensing. One pathway that is important for a productive T cell response is the activation of mitogen-activated protein kinase (MAPK) signaling. MAPK signaling is activated by a variety of external stimuli and plays an important role in regulating a variety of cellular processes such as growth,

differentiation, inflammation, and apoptosis. Activation of this cascade also occurs directly downstream of the TCR. When the TCR is engaged, the tyrosine kinase zeta chain associated protein of 70 KDa (ZAP70) is recruited to the TCR/CD3 complex, where it then acquires its own tyrosine kinase ability. The adapter protein linker of activated T cells (LAT) is phosphorylated by ZAP70, allowing it to bind another adapter protein, Grb-2. Grb-2 recruits Sos, a Ras GTP/GDP exchange factor, which then activates the Ras-MAPK pathway (Ashwell 2006).

There are three major groups of MAP kinases in this pathway: extracellular signal regulated kinase (ERK) (Schaefer and Weber, 1999), p38 MAP kinase (Han and Ulevitch, 1999), and c-Jun NH2-terminal kinase (JNK) (Davis, 2000). All groups are activated by dual phosphorylation by different MAP kinase kinases (MKKs) which are downstream targets of MAP kinase kinase kinases (MKKKs). Several different signals activate these MKKKs. ERK activation is regulated by growth factors and ras signaling, while JNK and p38 are stress activated and regulated by rho GTPases. Therefore, ERK is usually associated with growth and survival, while JNK and p38 are associated with cellular stress and apoptotic pathways.

Although not traditionally thought of as nutrient sensors, there is evidence that these kinases can play a critical role in amino acid sensing. There are several groups who have found that MAP kinases are involved in the regulation of the neutral amino acid transporter SNAT2 surface expression and function in response to amino acids (Franchi-Gazzola 1999, Hyde 2007). This would indicate that MAP kinases can be one of the key

regulators of amino acid sensing and utilization.

Glutamine is the most abundant amino acid in serum and is therefore one of the best candidates for being a target for nutrient sensing. Since mTOR and GCN2 have been shown to be sensitive to specific amino acids, such as leucine and tryptophan, respectively, it is possible that glutamine can also be sensed by either of these proteins or by another mechanism. This is likely because glutamine is required in many cell types for growth and proliferation. This would indicate that there would have to be a sensing mechanism put into place to tightly monitor extracellular glutamine concentrations in order for cells to react accordingly. In this chapter, we examined potential glutamine sensors in primary T cells in order to further understand how glutamine metabolism is regulated.

Materials and Methods

Reagents

Glutamine free RPMI (HyClone) was supplemented with Pen/Strep, 10 mM HEPES, 55 µM β-mercaptoethanol and 10% FBS. Glutamate free RPMI was made in the lab and supplemented as previously described. Purified CHOP protein was purchased from Santa Cruz.

Antibodies and Inhibitors

Rapamycin, an inhibitor of mTOR, was purchased from Alexa Biomedical and used at a concentration of 40 ng/ml. The MEK1 inhibitor, PD98059, p38 inhibitor SB203580, and the PI3K inhibitor, LY294002 were purchased by BioMol (Plymouth Meeting, PA) and

used at concentrations of 40µM, 20µM, and 10µM, respectively. Antibodies against 4EBP1, p70S6K, eIF2α, and ERK were purchased from Cell Signaling Technology (Beverly, MA). Anti-CHOP was purchased from US Biological (Swampscott, MA).

Analysis of Protein Expression

To examine ERK phosphorylation, T cells were allowed to incubate in glutamine free conditions for 2 hours prior to stimulation in order to allow them to recognize the change in the glutamine concentration. T cells were then stimulated with 10 µg/ml each of anti-CD3 and anti-CD28 on ice for 30 minutes. The secondary cross-linking antibody, goat anti-hamster IgG was added, then samples were transferred to 37°C for 0, 2, and 5 minutes. Stimulation was stopped with the addition of 10 volumes of cold PBS. For all other experiments, cells were stimulated overnight in RPMI containing anti-CD3/ anti-CD28 conjugated beads as previously described in Chapter 1. Cells were lysed in RIPA buffer (1% Nonidet P40, 0.5% sodium deoxycholate, 0.1 % SDS, 10 mM NaF, 1 mM Na_3VO_4 and 1 mM PMSF in PBS) and resolved on 12% polyacrylamide gels and transferred to nitrocellulose membrane. Lysates of $1x10^6$ T cell equivalents were loaded for each sample. Blots were blocked in PBS/0.1% Tween containing 5% nonfat dry milk and probed with primary antibody at 1:1,000. HRP conjugated anti-rabbit and anti-mouse IgG, (BioRad, Hercules, CA) was used at 1:10,000 as the secondary antibody. Protein bands were visualized using SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL). The blots were routinely stained with Ponceau Red to ensure equal loading as well as even transfer.

Results

Glutamine is not sensed by mTOR or GCN2

mTOR is a known amino acid sensor, especially for branched chain amino acids. Previous studies have demonstrated that mTOR signaling is inhibited in the absence of leucine. We therefore wanted to determine if mTOR was able to sense glutamine in T cells. Cells were stimulated overnight using anti-CD3/anti-CD28 bead-linked antibodies in the presence of glutamine or under glutamine-free conditions and lysates were prepared. mTOR activity was assessed by levels of phosphorylation of 4EBP1. Western blot analysis revealed that glutamine depletion did not affect 4EBP1 phosphorylation (Figure 11). To ensure that phosphorylation of 4EBP1 was mTOR dependent, T cells were stimulated in complete medium in the presence of the mTOR inhibitor rapamycin which caused a near complete block of 4EBP1 phosphorylation. From this result we can conclude that in T cells, glutamine is not sensed by mTOR.

Next, we wanted to examine whether glutamine may be sensed by GCN2. Previous studies have shown that GCN2 is activated when cells are depleted of tryptophan; therefore, we wanted to see if glutamine depletion also affected GCN2 activation. Using CHOP as an indicator of GCN2 activity, we found that when T cells were stimulated, CHOP was expressed regardless of whether glutamine was present (Figure 12). Although T cell activation induced CHOP expression, the presence of glutamine did not affect the intensity of expression.

Because T cell activation strongly induced CHOP, we decided to examine other proteins

Stimulated:	-	-	+	+	+
Glutamine:	+	-	+	-	+
Rapamycin:	-	-	-	-	+

Phospho-4EBP1

← 20kD

Figure 11. *Glutamine starvation does not affect mTOR activation.* T cell lysates were produced from cells stimulated with anti-CD3/ anti-CD28 conjugated beads for 24 hours in RPMI containing 2 mM glutamine or in glutamine-free RPMI. Rapamycin was added at 40 ng/ml as an inhibitor of mTOR dependent 4EBP1 phosphorylation.
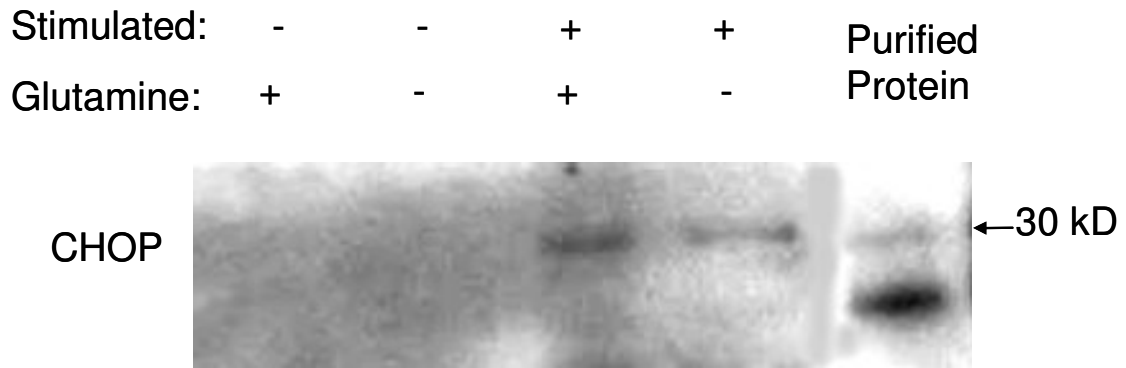
Figure 12. *GCN2 activity is independent of glutamine.* T cell lysates were produced from cells stimulated with anti-CD3/ anti-CD28 conjugated beads for 24 hours in RPMI containing 2 mM glutamine or in glutamine-free RPMI. GCN2 induction was measured by CHOP expression, a downstream target of GCN2.

which were more proximal to GCN2 to get a better sense of whether this induction was specific for GCN2 activity. eIF2α is a direct target of GCN2 and may therefore act as a better indicator of GCN2 activity than CHOP. When the same conditions were used to study eIF2α, we found that as glutamine depletion did not induce phosphorylation (Figure 13). These results suggest that although GCN2 senses overall amino acid availability, it may not sense glutamine depletion alone within 24 hours of stimulation.

MAPK signaling is sensitive to glutamine levels

Since the two most characterized amino acid sensitive pathways were not glutamine sensors, we then turned to pathways that were not known to be amino acid sensors per se but had been implicated as being potential regulators of amino acid transport and metabolism. MAP kinases are well established to be important for many cellular processes required for growth and differentiation, as well as apoptosis. These kinases regulate activity of other signaling molecules known to be affected by amino acid availability, such as GCN2 components (Fan 2002, Wang 1996, Hai 1999), and have been implicated as targets of amino acid transporter signaling (Franchi-Gazzola 1999). The MAPK cascade is activated immediately downstream of the T cell receptor. To determine whether glutamine depletion plays a role in MAPK signaling, cells were cultured in the absence of glutamine for 2 hours to deplete glutamine stores, and then stimulated for up to 5 minutes in the presence or absence of glutamine. ERK phosphorylation was measured as an indication of MAPK activity. We found that phosphorylation of ERK was decreased in the absence of glutamine, indicating that ERK signaling may be regulated by glutamine (Figure 14).
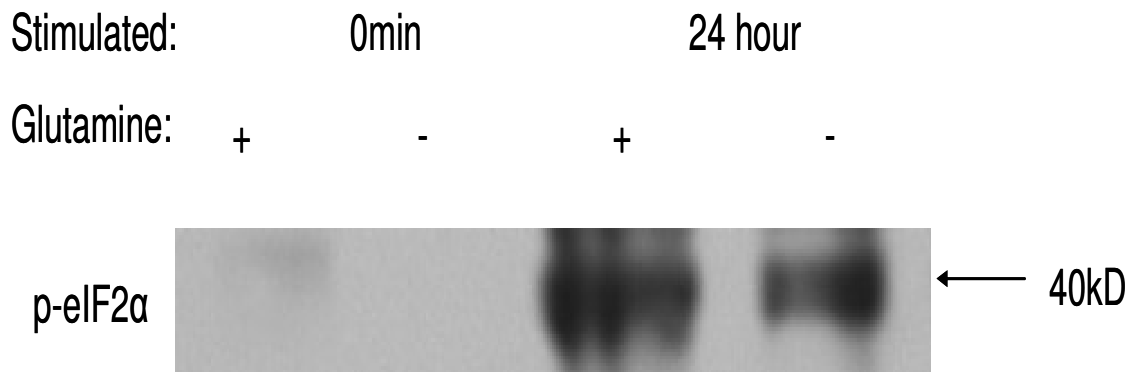
Figure 13. *Glutamine starvation does not inhibit eIF2α phosphorylation*. T cell lysates were produced from cells stimulated with anti-CD3/ anti-CD28 conjugated beads for 24 hours in RPMI containing 2 mM glutamine or in glutamine-free RPMI. GCN2 induction was measured by eIF2α phosphorylation, a downstream target of GCN2.
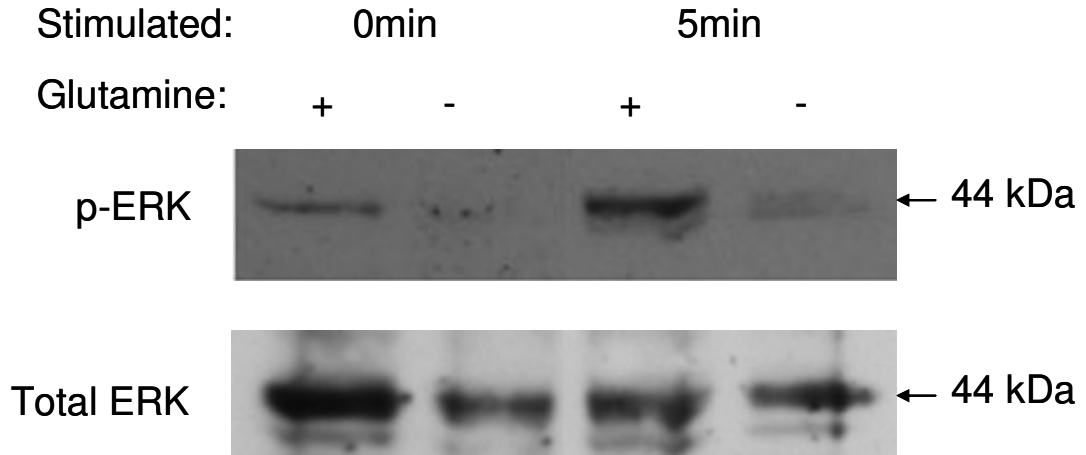
Figure 14. *MAPK signaling is regulated by glutamine*. Purified T cells were incubated for 2 hours in glutamine-free RPMI to deplete intracellular glutamine stores. T cells were then stimulated for 0 and 5 minutes using anti-CD3/anti-CD28 crosslinking antibody in the presence or absence of 2 mM glutamine. MAPK activation was determined by ERK phosphorylation.

Regulation of Activation Markers

As an alternative way of examining what pathways may be sensitive to glutamine, we used inhibitors of key signaling pathways to try to mimic the effects of glutamine depletion. To address this, we compared surface expression of CD25, CD44, CD69 and 4F2 (as described in Chapter 1) in glutamine depleted T cells versus T cells grown in various inhibitors (Figure 15). Although glutamine depletion has some affect on CD25, it seems to regulate CD25 expression differently than PI3K, mTOR or MAPK. This would suggest that although glutamine depletion affects ERK signaling, it plays a different role in early activation than ERK. We also noted that glutamine depletion led to a slight enhancement in CD69 surface expression which is not seen in the presence of any of the inhibitors. This finding would suggest that glutamine depletion cannot be mimicked by the loss of a single signaling pathway, but likely results in a more complex regulation of signaling molecules.

Conclusion

Taken together, these data imply that in fact glutamine is not required simply as a metabolic source but is also involved in cellular signaling. We have shown that although glutamine is not sensed by traditional sensing pathways such as GCN2 and mTOR, glutamine has a pronounced effect on MAPK activity. In the next chapter, we aim to determine how glutamine transport is regulated during T cell activation and which transporters capable of carrying glutamine are present in T cells. When T cells are activated, they need to increase import of many nutrients such as amino acids in order to facilitate proper immune response. We hypothesize that T cells would differentially

regulate amino acid transporters in order to increase transport of certain amino acids while inhibiting the transport of others.
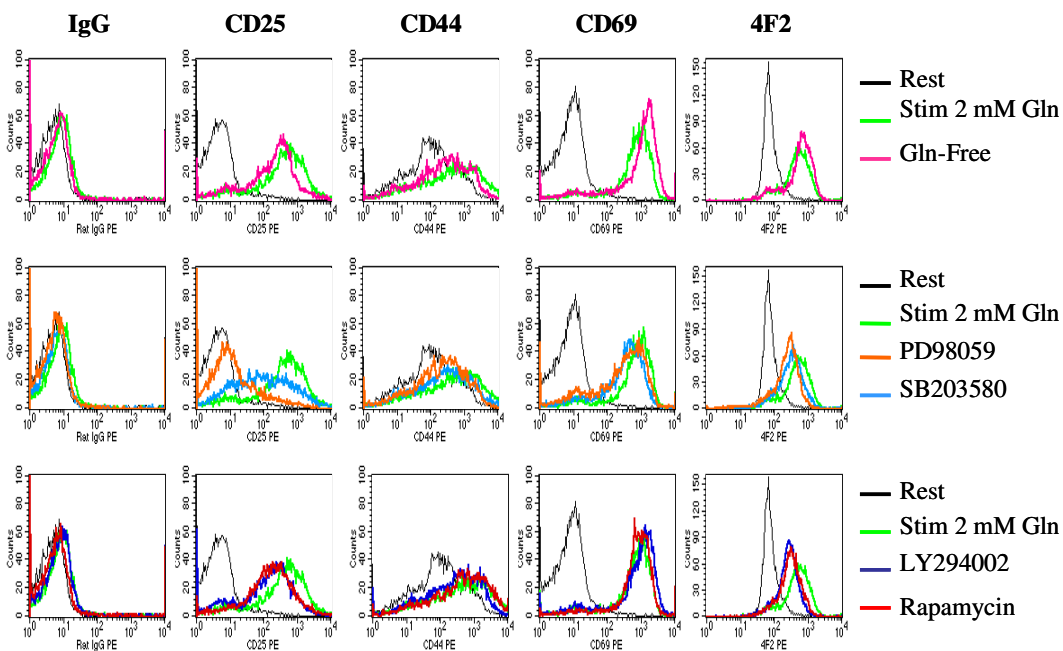
Figure 15. *Early activation markers are differentially expressed.* Splenic T cells were purified and stimulated for 20 hours using anti-CD3/ anti-CD28 conjugated beads. Activation marker profiles of T cells cultured in 2mM glutamine was compared to those cultured in either glutamine-free RPMI (A), RPMI containing MEK1 inhibitor PD98059 (B), P38 inhibitor SB203580 (B), PI3K inhibitor LY294002 or mTOR inhibitor rapamycin. T cells were stained with PE-linked rat IgG, CD25, CD44, CD69 and 4F2 then analyzed by flow cytometry.

Chapter 3: Regulation of Glutamine Transport

Transport of Amino Acids

In previous chapters, we addressed the idea that amino acids may modulate T cell signaling during activation. However, the presence of these amino acids is also necessary to compensate for the enhanced metabolic needs of rapidly dividing T cells. We were particularly interested in glutamine transport because glutamine is known to be a primary fuel source in activated T cells. To investigate this, we wanted to study how amino acids were transported across the cell membrane and what were the key transporters involved during T cell activation.

Amino acid transporters are divided into a series of systems based on their mode of transport and the types of cargo. For example, glutamine transport is known to take place in system A, N, ASC and L (Table 1 and Figure 16). Systems A, N, and ASC are sodium dependent transport systems that are capable of taking up neutral amino acids, while system L is sodium independent. Systems A and N have high sensitivity to pH with optimal transport activity at pH 7-8 (Baird 2006) and because of their similarities are often grouped into one family of transporters called SNATs (sodium coupled neutral amino acid transporters). System N consists of SNAT3 and SNAT5 which are key mediators of glutamine transport that are known to be expressed in many cell types (Baird 2004). System A consists of SNAT1, SNAT2 and SNAT4 and is known to take up a variety of neutral amino acids. These transporters can also transport glutamine but with a lower affinity (Baird 2004). System L consists of LAT1 and LAT2, which are transporter proteins that are linked to 4F2 as the heavy chain. The 4F2 protein is known
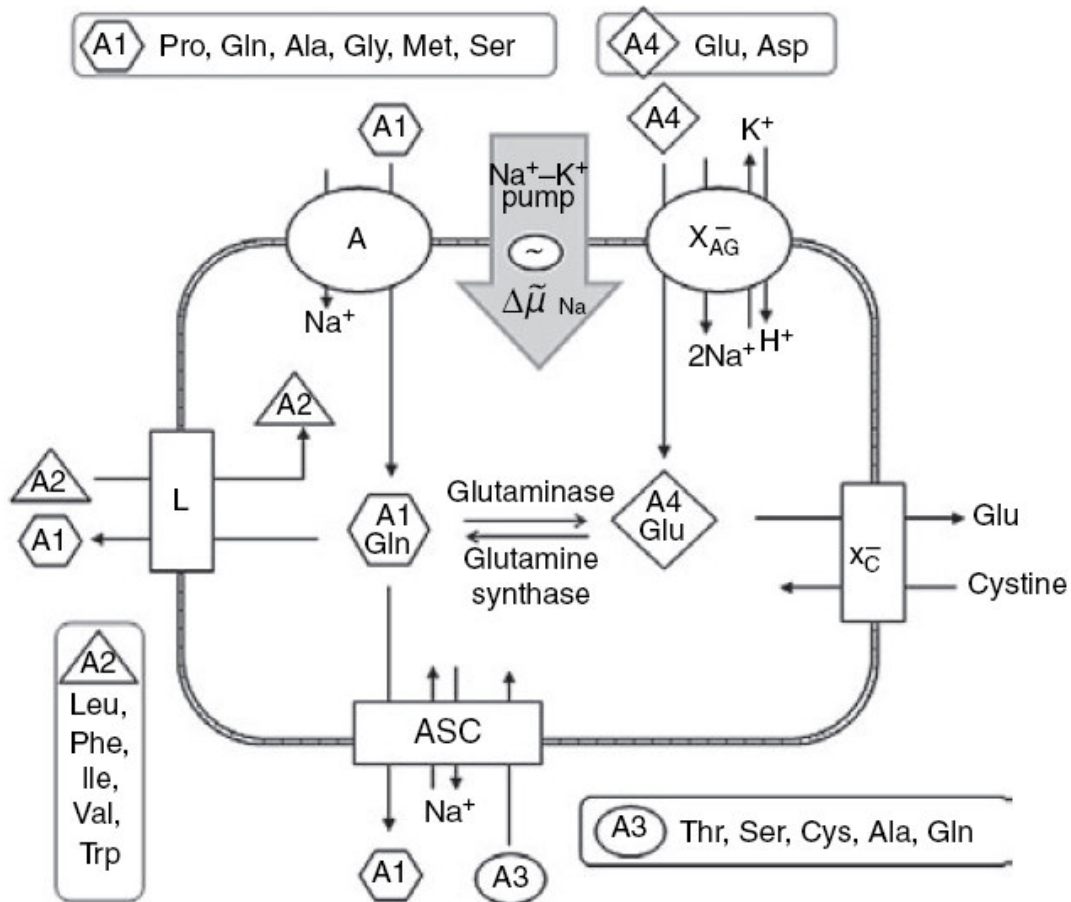
Figure 16. *Glutamine Transport*. Glutamine can cross the plasma membrane through various amino acid transporters. Amino acid transporters are divided into a series of systems based on their mode of transport and the types of cargo. Glutamine transport can take place in systems A, N, ASC and L depending on cell type. Systems A, N, and ASC are sodium dependent transport systems that are capable of taking up neutral amino acids, while system L is sodium independent. Systems A and N have many similarities and therefore are often grouped into one family of transporters called SNATs (sodium coupled neutral amino acid transporters). System L transporters import a variety of neutral amino acids but exports glutamine. Glutamine transport via system ASC is bidirectional (This figure was taken from Franchi-Gazzola 2006).

Table 1: Key Glutamine and Glutamate Transporters

System	Gene Name	Protein Name	Accession Number	Transport Type
Glutamine Transporters				
System A	SLC38A1	SNAT1	NM_030674	Cotransport/ Na+
System A	SLC38A2	SNAT2	NM_018976	Cotransport/ Na+
System A	SLC38A4	SNAT4	NM_018018	Cotransport/ Na+
System N	SLC38A3	SNAT3	NM_006841	Cotransport/ Na+, antiport/ H+
System N	SLC38A5	SNAT5	NM_033518	Cotransport/ Na+, antiport/ H+
Glutamate Transporters				
System X^-_{AG}	SLC1A3	EAAT1	NM_005628	K+ antiport
System X^-_c	SLC7A11	Xct	NM_014331	Glutamate/cystine exchanger

to associate with many transporters and is thought to act as a chaperone that is important for membrane targeting (Verrey 2003). Lastly, system ASC is also a sodium dependent neutral amino acid transporter that is capable of transporting glutamine across the plasma membrane. Although there are many transporters capable of transporting glutamine, it is unknown which are the dominantly expressed in T cells. In this chapter, we examined glutamine utilization as well as transporter expression in order to determine how these processes are regulated during T cell activation.

Materials and Methods

[3]H Amino Acid Uptake Assay

[3]H-glutamine and glutamate were purchased from American Radiolabeled Chemicals. Columns were made for amino acid uptake using a 500µL centrifuge tube containing a bottom layer of 25 µL of 20% perchloric acid/8% sucrose, followed by a middle layer of 200µL of 2-bromododecane . The top layer consisted of 100 µL of RPMI containing 2 µCi of [^{3}H]-glutamine or [^{3}H]-glutamate. Cells were stimulated overnight with bead-linked anti-CD3/anti-CD28 antibodies in RPMI. For some experiments, RPMI was supplemented with inhibitors (see Table 2). PD98059, SB203580 and LY294002 were purchased from BioMol (Plymouth Meeting, PA) and used at 40 µM, 20 µM, and 10 µM, respectively. Rapamycin was used at a concentration of 40 ng/ml (Alexa Biochemicals, San Francisco, CA). Cells were washed in PBS, and resuspended in glutamine free RPMI at 2.5×10^7/ml. 2.5×10^6 cells were then added to the radioactive layer and allowed to incubate at room temperature for 10 minutes. Cells were isolated from the radioactive layer by centrifugation into the acidic layer. Reaction tubes were then frozen on a dry

ice/ethanol bath and the acidic layer containing the T cells was cut off and analyzed. Uptake was measured by scintillation using a Microbeta Trilux scintillation counter (Wallac, Turku, Finland). Uptake assays using EL4 cells had slight variations. Because EL4 cells took up far more glutamine or glutamate than primary T cells, only 1×10^6 cells were used per reaction and 1µCi of radioactivity was used. All other procedures were done according to the above protocol.

RNA Isolation

Primary murine T cells were isolated from spleens of C57BL/6J mice (as described previously). T cells were either stimulated with anti-CD3/ anti-CD28 conjugated beads or with IgG conjugated beads for designated time points. After stimulation, RNA was extracted using the NucleoSpin RNA II kit following the manufacturer's instructions (Macherey-Nagel, Bethlehem,PA).

Reverse Transcriptase PCR

Synthesis of cDNA was performed using the iScript cDNA synthesis kit (BioRad) according to manufacturer's instructions. PCR reaction was as follows:

25°C for 5 min, 42°C for 30 min, 85°C for 5 min

Quantitative Real Time PCR

Real time PCR performed using the iQ SYBR Green Supermix (BioRad) following the manufacturer's instructions. Gene specific primers were made using the Beacon Designer 4 software (Premier Biosoft International). Each duplicate sample consisted of 300nM primer pair mix, 1µl cDNA template, and 12.5 µl of iQ Sybr Green supermix. Sample volume was brought up to 25µl using DEPC treated water. Amplification was performed using the iCycler iQ Multicolor Real-Time PCR Detection System (BioRad) using the

following cycling conditions:

95°C 3 min cycled once followed by 40 cycles of: 95°C for 15 sec, 57°C for 30 sec, and 72°C for 30 sec. Melt curve analysis was performed by running one cycle at 95°C for 1 min followed by one cycle of 55°C at 1 min. The temperature was then elevated by 0.5°C 80 times until it reached 95°C.

Relative mRNA abundance was normalized to mRNA levels of 18S at equivalent time points. Fold increase was presented as relative increase exceeding resting expression.

Results

Transport of glutamine and glutamate during T cell activation

We have previously confirmed that T lymphocytes require glutamine for T cell proliferation. To get a quantitative measurement of glutamine and glutamate utilization during T cell activation, uptake of radio-labeled amino acid was measured in the constitutively activated T lymphoma cell line EL4. Glutamine has been shown to be important in tumor progression. Studies have also found that glutamine supplementation in cancer patients has decreased tumor growth presumably by increasing the efficiency of the immune system (Klimberg 1996). By first investigating glutamine/glutamate utilization in these tumorigenic cells first, we could use them as a control to validate our experimental technique as well as to determine the sensitivity of the assay.

We first wanted to determine how much glutamine relative to glutamate was taken up by these cells so glutamine/glutamate uptake was measured (Figure 17). Data analysis revealed that glutamine influx in EL4 cells was almost 10 times faster than that of
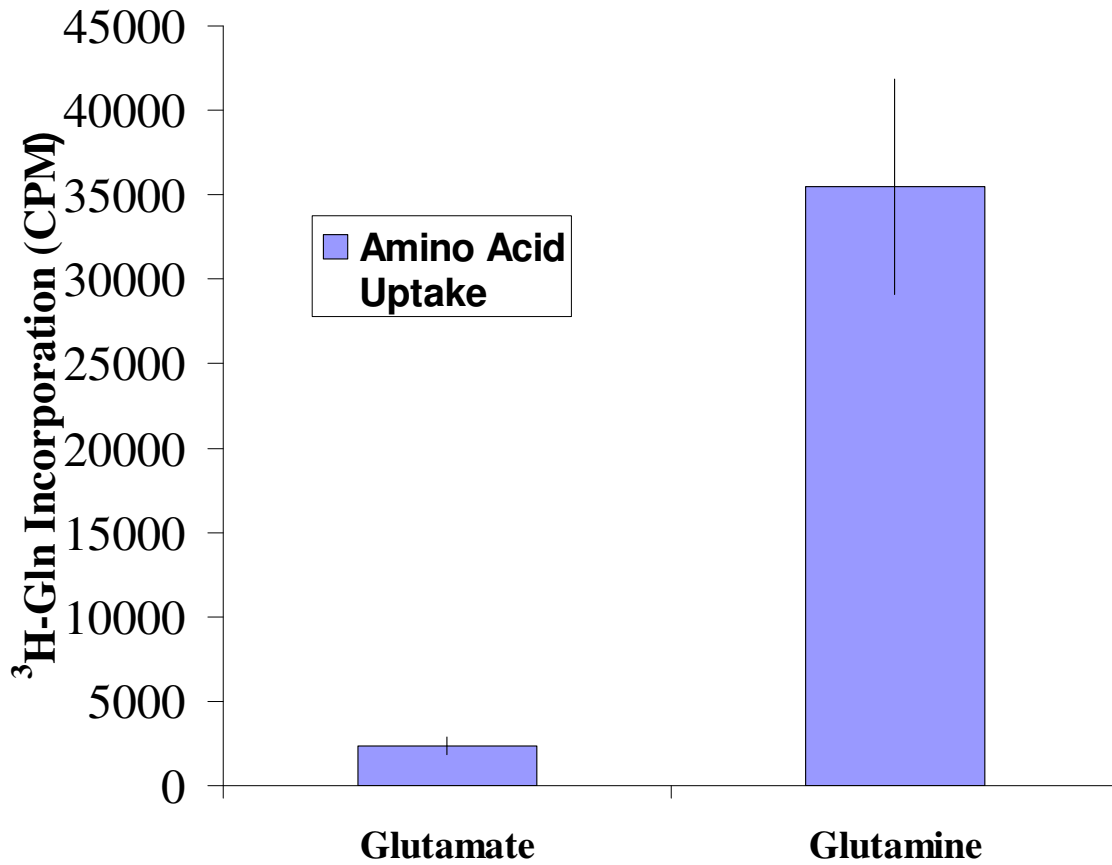
Figure 17. *Mouse T lymphoma cells rapidly import glutamine and glutamate.* EL4 cells were grown overnight in RPMI containing glutamine then washed and resuspended in glutamine free RPMI at a concentration of 1×10^7 cells per ml. Uptake rates of radio-labeled glutamine and glutamate were measured after 2 minute incubations by scintillation counting. Error bars represent ± standard error of samples analyzed in triplicate.

glutamate. We next wanted to examine glutamine/glutamate uptake in resting and stimulated T cells for comparison (Figure 18). Resting T cells had equivalent uptake rates of glutamine and glutamate. The relative influx of glutamine in cells stimulated for 48 hours was 10 times higher than resting cells. By contrast, glutamate uptake in stimulated cells was only 2 times greater than resting T cells. Thus, glutamine uptake is induced much more than glutamate uptake by T cell activation.

From previous experiments, we determined that glutamine may regulate the ERK pathway. We next wanted to determine how ERK and other pathways could affect glutamine flux. To address this, we treated T cells with various inhibitors of well known signaling pathways and measured their effects on glutamine uptake (Figure 19 and 20). The signaling molecules that we chose to inhibit were PI3K, mTOR, p38 and ERK (see Table 2). Inhibitors of any of these pathways reduced glutamine uptake but inhibition by the MEK1 inhibitor PD98059 caused the greatest and most consistent effect on uptake. This indicates that ERK signaling regulates glutamine uptake. The PI3K inhibitor LY294002 and rapamycin induced mTOR inhibition results suggest that glutamine uptake was inhibited, but the experiments need to be repeated.

Regulation of Amino Acid Transporter Expression

We have shown that T cells rapidly increase uptake of amino acids when stimulated. We wanted to determine which amino acid transporters were up-regulated during T cell activation, with an emphasis on transporters that were capable of transporting glutamine and glutamate. In order to characterize transporters that were involved in amino acid
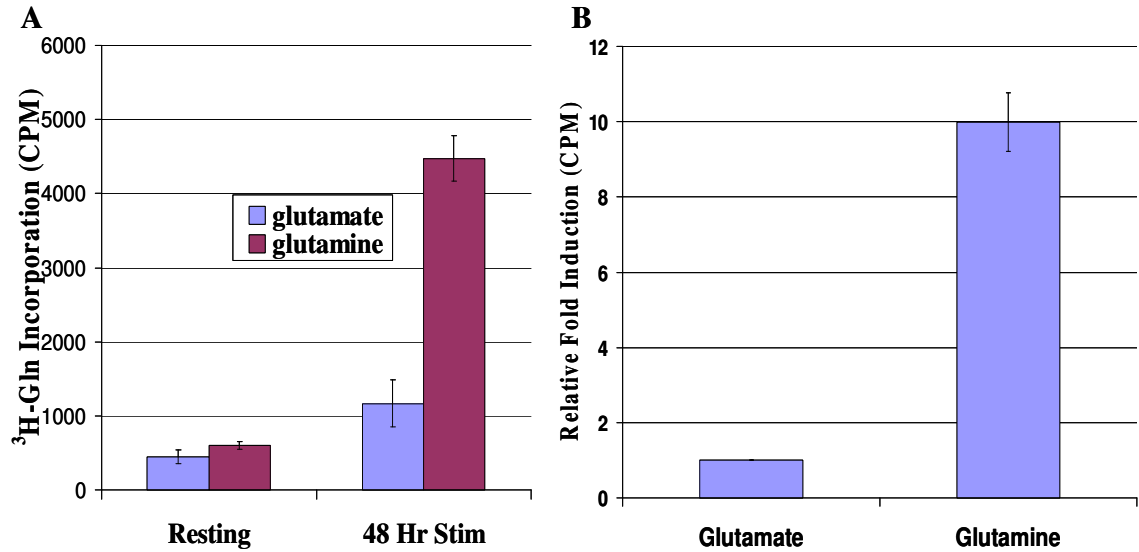
Figure 18. *Mouse primary T cells import glutamine faster than glutamate.* Splenic T cells were purified and stimulated with anti-CD3/anti-CD28 antibody for 24 hours in RPMI containing glutamine then washed and resuspended in glutamine free RPMI at a concentration of 2.5 x10^7 cells per ml. Uptake rates of radio-labeled glutamine and glutamate were measured after 10 minute incubations by scintillation counting. Graphs represent relative uptake rate (A) and fold difference based on molar concentration of amino acid (B). Error bars represent ± standard error of samples analyzed in triplicate.

Table 2: Signaling Inhibitors

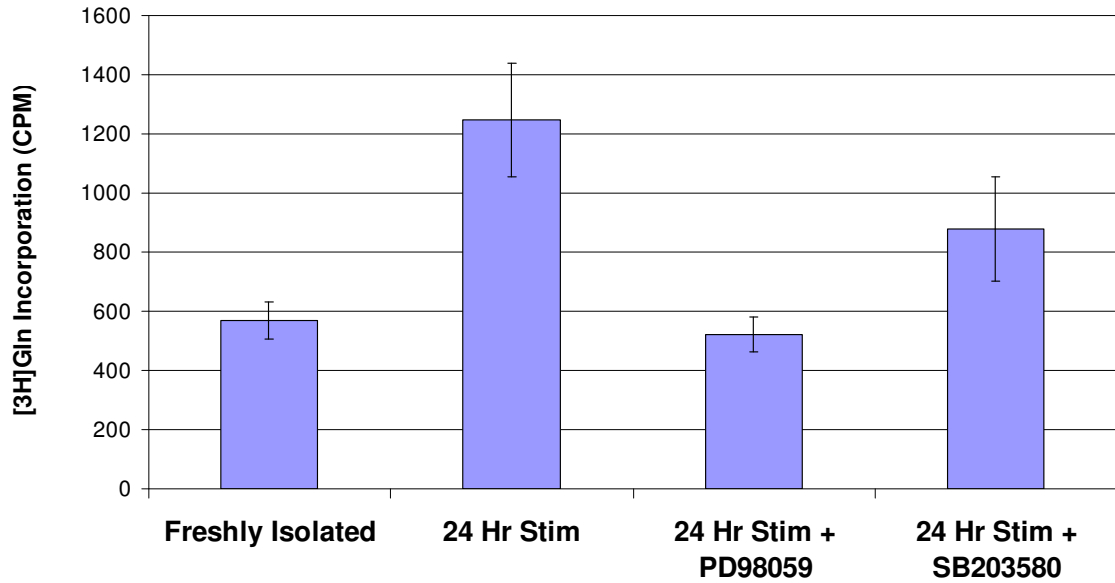| Inhibitors	Target
Rapamycin	mTOR
PD98059	MEK1
SB203580	p38
LY294002	PI3K

Figure 19. MAPK *inhibitors regulate glutamine uptake.* Splenic T cells were purified and stimulated with anti-CD3/anti-CD28 antibody for 24 hours in RPMI containing glutamine supplemented with either the MEK1 inhibitor PD98059 or the P38 inhibitor SB203580, then washed and resuspended in glutamine free RPMI at a concentration of 2.5 x10^7 cells per ml. The uptake rate of radio-labeled glutamine was measured after 10 minute incubations by scintillation counting. Error bars represent ± standard error of samples analyzed in triplicate.
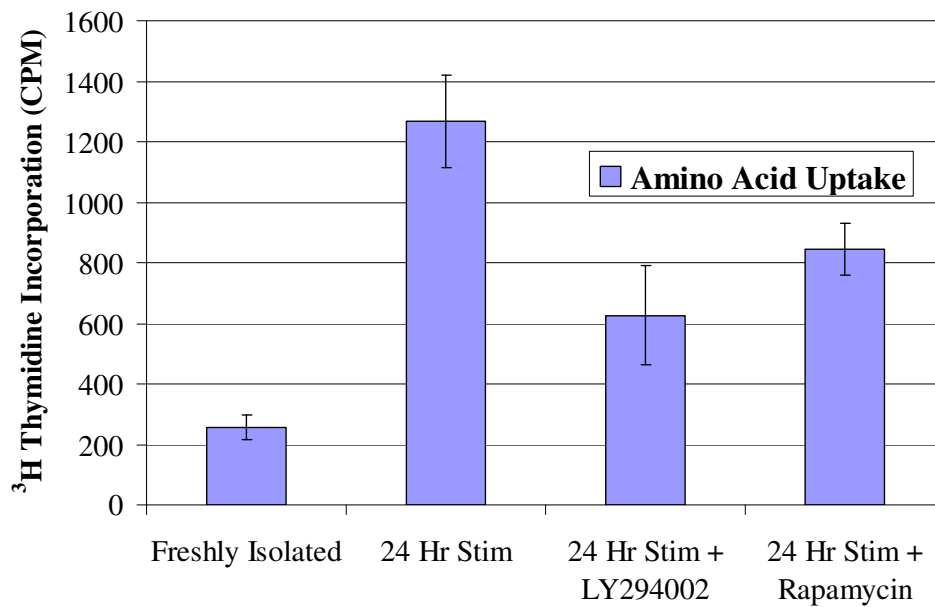
Figure 20 G*lutamine uptake is regulated by mTOR and PI3K signaling.* Splenic T cells were purified and stimulated with anti-CD3/anti-CD28 antibody for 24 hours in RPMI containing glutamine supplemented with either the mTOR inhibitor rapamycin or the PI3K inhibitor LY294002, then washed and resuspended in glutamine free RPMI at a concentration of 2.5 x10^7 cells per ml. The uptake rate of radio-labeled glutamine was measured after 10 minute incubations by scintillation counting. Error bars represent ± standard error of samples analyzed in triplicate.

trafficking, we examined transporter expression on select amino acid transporters during T cell activation. We designed primers specific for a variety of amino acid transporters and were able to use these primers to measure expression in resting and stimulated T cells. Expression of the ribosomal RNA 18S was used as a housekeeping gene as stimulation had little effect on its expression. T cells were allowed to rest with beads conjugated with IgG antibody or induced to activate with anti-CD3/CD28 beads for 3 hours, 8 hours and 24 hours. RNA was extracted and cDNA was synthesized. Quantitative PCR analysis revealed that when T cells are activated, they selectively up-regulate expression of some amino acid transporters. While some were highly up-regulated after stimulation, others remained at basal levels. We also observed that these transporters were up-regulated at different times after stimulation. For instance, expression of the cationic amino acid transporter CAT1 was the highest expressing transporter as expression was significantly increased as early as 3 hours after stimulation and continued to increase after 24 hours of stimulation. In contrast, it's closely related family member CAT3, did not induce expression until 24 hours of expression (Figure 21). We also discovered that CAT2b, a transporter known to be highly expressed in macrophages was not expressed at all in murine T cells (Figure 22).

We then wanted to concentrate on glutamine transporters, so expression of select SNAT family transporters was examined. Like other amino acid transporters, we found that these transporters are also up-regulated during stimulation. Quantitative PCR analysis revealed that when T cells are activated, expression of the System A transporter, SNAT1 is up-regulated after 8 hours and remains for at least 24 hours (Figure 23). Another

50
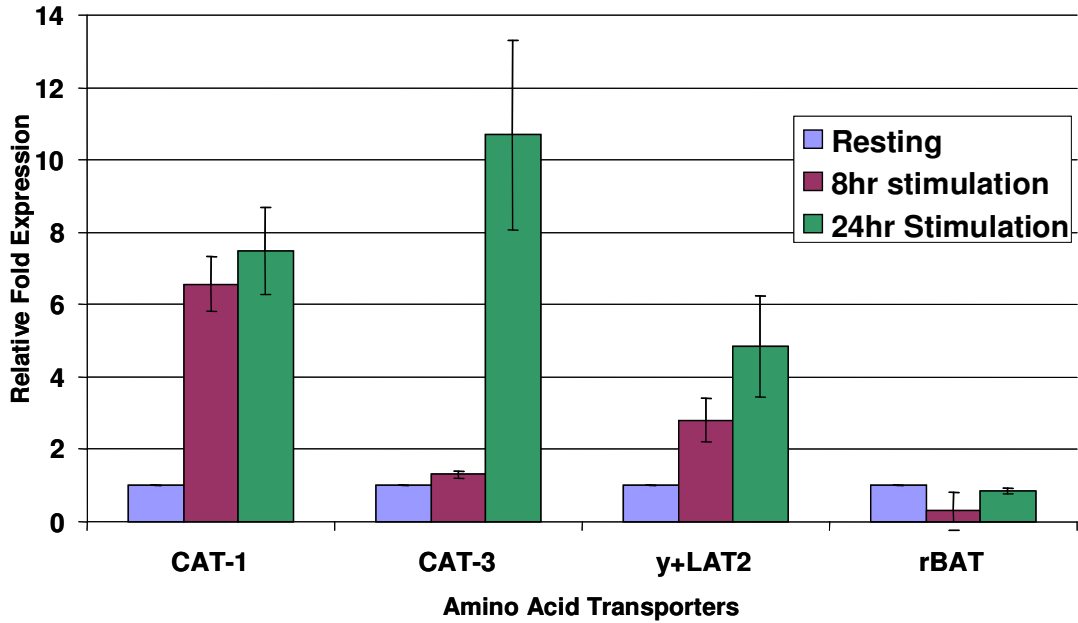
Figure 21. *Primary T cells upregulate amino acid transporters upon activation*. Splenic T cells were purified then stimulated with anti-CD3/anti-CD28 conjugated beads for 0, 8 and 24 hours then RNA was extracted and cDNA was synthesized. Expression of amino acid transporters were measured by real time PCR analysis using 18S as a housekeeping gene. Expression levels of CAT1, CAT3 and y+LAT2 and rBAT were measured. Error bars represent ± standard error of samples analyzed in triplicate.

Figure 22. *CAT1 and CAT2b are differentially expressed.* Splenic T cells were purified then stimulated with anti-CD3/anti-CD28 conjugated beads for 0 and 24 hours then RNA was extracted and cDNA was synthesized. Expression of CAT1 and CAT2b transporters were measured by PCR analysis. Act Mϕ = Activated Macrophages.
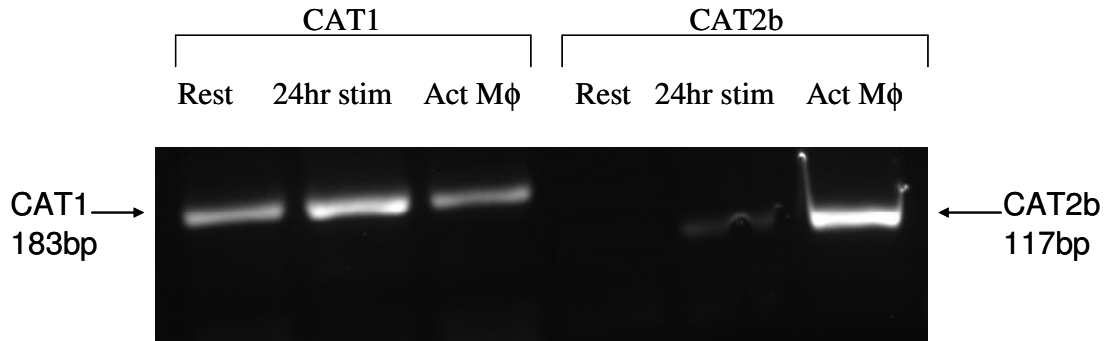
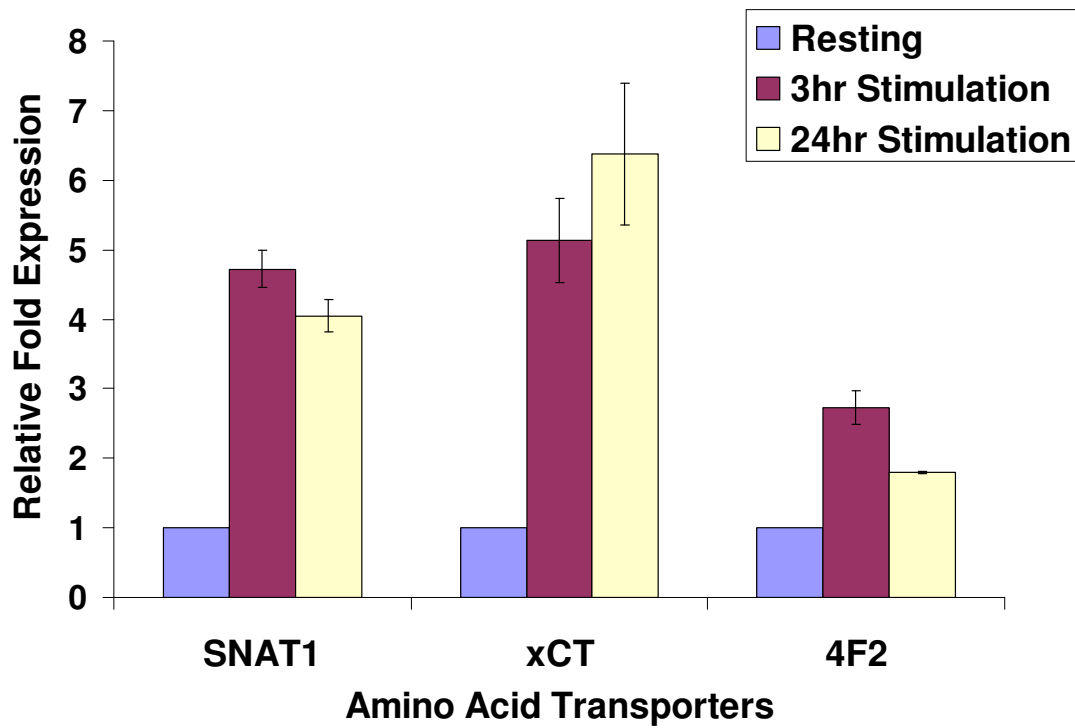Figure 23. *Glutamine and glutamate transporters are upregulated during activation.* Splenic T cells were purified then stimulated with anti-CD3/anti-CD28 conjugated beads for 0, 3 and 24 hours then RNA was extracted and cDNA was synthesized. Expression of amino acid transporters were measured by real time PCR analysis using 18S as a housekeeping gene. Error bars represent ± standard error of samples analyzed in triplicate.

glutamine transporter from system N, SNAT3 was up-regulated at the RNA level after 8 hours of stimulation (Figure 24). However, RNA levels declined substantially by 24 hours. Basal levels of SNAT3 expression were so low that quantitative PCR was not feasible.

We knew from previous studies that glutamate supplementation in glutamine depleted cells could not rescue the proliferative defect in these T cells. We also found that glutamate transport is much lower than glutamine transport in activated T cells. We hypothesized that glutamine was metabolized to glutamate then rapidly shuttled out of the cell. To test this hypothesis, we examined the expression of glutamate transporters upon T cell stimulation. Although glutamate transport has been extensively studied in cells of the central nervous system, its transport in T cells are less characterized. In neuronal cell types, glutamate is transported via system X_{ag} and X_c^-. System X_{ag} consists of sodium dependent transporters called EAATs (excitatory amino acid transporters) that are responsible for importing extracellular glutamate. The transporter protein for system X_c^- is xCT, a cystine/glutamate exchanger, which takes up cystine in exchange for glutamate efflux. Some studies have found that T cells have very weak transport activity for cystine through this transporter regardless of whether the cell is stimulated or at rest (Gmunder 1991). To determine if we could see the same results in our hands, we examined xCT expression upon stimulation. Unlike in previous studies, we observed a five fold increase in xCT expression after 8 hours of stimulation (Figure 23) suggesting that glutamate export may be upregulated in activated T cells.
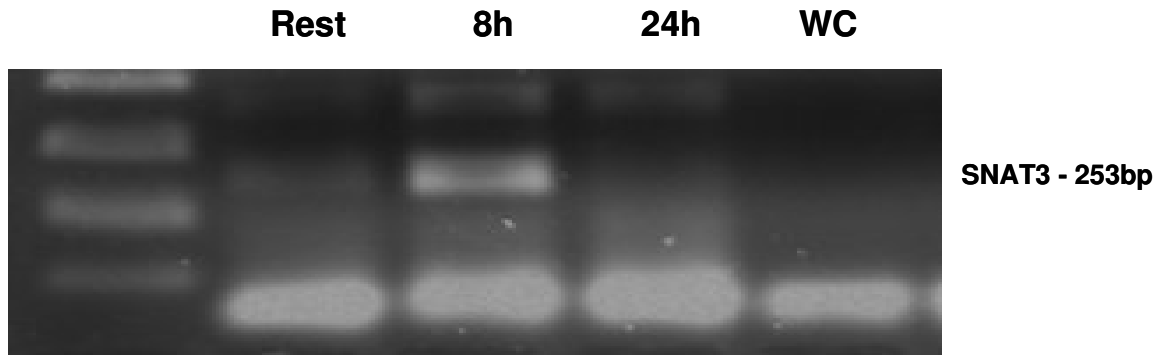
Figure 24. *SNAT3 is upregulated during T cell activation.* Splenic T cells were purified then stimulated with anti-CD3/anti-CD28 conjugated beads for 0, 8 and 24 hours then RNA was extracted and cDNA was synthesized. Expression of the glutamine transporter SNAT3 was measured by PCR analysis.

Conclusion

Taken together, these results indicate that amino acid transporters are differentially regulated during T cell activation. T cells regulate whether these transporters should be expressed as well as when. This implies that in certain transporters may be necessary during the initial stages of activation while others may play a more active role later in this process. Further studies would include assessing transporter expression over longer periods of time greater than 24 hours. We may observe that although both system A and System N transporters are both necessary within the first 8 hours of stimulation, one may take on a much more substantial role later. For example, we observe a wave of SNAT3 RNA expression in which it goes from almost no expression at rest, being expressed at 8 hours then decreasing again after 24 hours. There may be another wave of SNAT3 expression later in T cell activation, or it may only be needed at the earliest activation phase. Additionally, we observed that XCT, a transporter known to export glutamate is upregulated upon T cell stimulation. This supports our hypothesis that glutamine import may become more important also as a source of glutamate that is rapidly exported, perhaps as a mechanism of importing cystine or other amino acids.

Glutamine Transporters as Sensors

Previous studies have shown that some nutrient sensors are actually transporters or receptors for that particular nutrient. We speculated that these transporters may be key regulators of T cell activation both by regulating amino acid flux as well as potentially acting as receptors for T cell signaling pathways in response to amino acid availability. In *Sacchromyces cerevisiae*, transporters are direct sensors of amino acid availability. These

transporters are transcriptionally regulated depending on substrate availability in the extracellular environment (Horak 1997). In higher eukaryotes, glutamate receptors in astrocytes have been known to be sensors of extracellular glutamate concentrations in order to regulate excitatory transmission (Vermeiren 2005). Binding to these receptors promote recruitment of glutamate transporters to the cell surface facilitating glutamate influx.

It has been suggested that amino acid transporters could sense and subsequently regulate amino acid transport in mammalian cells (Hyde 2003). More recent work has suggested that the neutral amino acid transporter, SNAT2 is both a transporter and a sensor of amino acids (Hyde 2007). It was shown that amino acid starvation of several cell lines, such as muscle cells and fibroblast cells, lead to increases in MAPK activity, in particular ERK and JNK. The elevation in activity led to an up-regulation of SNAT2 expression as well as an increase in SNAT2 protein stability. SNAT2 expression was blocked by JNK inhibition further indicating that JNK signaling regulates SNAT2 expression. This group identified this transporter as a possible sensor because when SNAT2 was overexpressed, it decreased promoter activity in a SNAT2 reporter construct. Furthermore, when SNAT2 was knocked down, it increased endogenous SNAT2 reporter activity. This indicated that SNAT2 could negatively feed back on itself to repress its own promoter when amino acids are in sufficient supply.

There are other signaling pathways known to be sensitive to amino acid availability such as mTOR, which has also been implicated in regulating amino acid transporter

expression. Rapamycin treatment resulted in selective inhibition of ASCT2, LAT1 and 4F2 expression in human B cell and mouse T cell lines (Peng 2002). The Frauwirth lab has also found that 4F2 expression is inhibited by rapamycin in primary murine T cells (unpublished observations). Taken together, these findings suggest that many signaling pathways regulate amino acid transporters and may therefore play important roles in amino acid flux. Importantly, amino acid transporters may also utilize a feedback mechanism which may regulate cell signaling in response to transporter activity.

There are several signaling molecules that are capable of sensing amino acids or their metabolites. We are particularly interested in the mechanism by which the presence or absence of glutamine modulates these signaling pathways. There are several ways in which glutamine can affect signaling. One aspect may be in the way in which amino acids are taken up. When we addressed this possibility we found that glutamine uptake is impaired when key signaling pathways are inhibited. We also observed that glutamine depletion negatively affected ERK activity. By analyzing key amino acid transporter expression under glutamine starvation, we can gain more insight into the role it plays in T lymphocyte activation. To do that, it is necessary to investigate changes in amino acid transporter expression in the presence of inhibitors of key T cell signaling pathways such as the MAPK pathway and the mTOR pathways.

Several studies have been done on the effects of amino acid deprivation on transporter expression, but there is very little data which specifically examines the contribution of individual amino acids on cell signaling and how it relates to transporter activity. It has

been previously shown that when human fibroblast cells are amino acid starved, they activate JNK and ERK. This activation leads to up-regulation of neutral amino acid transporters from system A (Franchi-Gazzola 1999). However, glutamine deprivation alone was found to up-regulate neutral amino acid transporters and 4F2 when assessed in human BJAB B lymphoma cells and murine CTLL2 T cell lines (Peng 2002). In our study, data suggests that unlike complete amino acid starvation in muscle and fibroblasts, glutamine deprivation causes deactivation of ERK. Our data also shows that overall glutamine transporter expression may be regulated by ERK but we do not know specifically which transporters are affected. Future studies would involve determining whether glutamine starvation is truly acting separately from total amino acid starvation and through which transporters are these signaling pathways acting on in T cells.

Discussion

The goal of this study was to elucidate the importance of glutamine in T cell activation. We conducted a comprehensive survey of amino acid requirements in primary T cells. Depleting individual amino acids revealed that while many amino acids were not required for T cell activation, depletion of some amino acids caused a proliferative defect as well as decreased cytokine production. Of all the required amino acids, glutamine depletion caused the greatest effect on T cell activation. We found that T cells require glutamine for purposes other than as an energy source because supplementation of glutamate or any of its precursors was unable to compensate for the loss of glutamine. When glutamine is reduced, there is a dose-dependent response to proliferation, suggesting that glutamine may be acting as a sensing molecule in T cells. Although glutamine starvation results in defects in later activation events, early activation markers are unaffected suggesting that glutamine's regulation on T cell activation is downstream of these earlier signals.

Upon examining potential signaling pathways that could be regulated by glutamine availability, we found that the two most established amino acid sensors, mTOR and GCN2 were not sensing glutamine. However, MAP kinase signaling, particularly ERK, was suppressed by glutamine depletion. To further examine pathways that may be regulated by glutamine, we assessed expression of key early activation markers in the presence of inhibitors of key signaling pathways. Inhibition of PI3K, mTOR, ERK and p38 had adverse effects on activation marker expression. Expression of CD25 was affected by all inhibitors as well as under glutamine-free conditions. However, glutamine depletion had a milder effect on expression of these markers as compared to the signaling

inhibitors. The fact that glutamine starvation had a different effect than any of the inhibitors, suggests that inhibition of any of these pathways could not mimic glutamine starvation and that glutamine may be working through multiple pathways. We then wanted to study how glutamine is transported and how this transport is regulated during T cell activation. Glutamine is rapidly shuttled into the cell upon stimulation as compared to glutamate. However, glutamine transport is negatively affected by ERK inhibition, suggesting that while glutamine may be regulating ERK signaling, ERK may regulate transport of glutamine. Upon surveying amino acid transporter expression, we found that transporters are differentially expressed during T cell activation. In particular, the glutamine transporter SNAT1 is continuously expressed up to 24 hours of expression while its closely related family member, SNAT3 is expressed at 8 hours but declines after 24 hours.

Taken together, these findings indicate that glutamine in fact may be a key signaling molecule in regulating T cell activation. There are some groups whose data support the idea that amino acids can act as signals and that transporters of these amino acids can possibly sense certain amino acids (Hyde 2007). Although our data suggest glutamine may be a signaling molecule, further studies would have to be implemented to confirm this. For example, the mechanism by which glutamine acts as a signaling molecule is unknown. It could bind specifically to an amino acid sensor, a cell receptor or an amino acid transporter. We show that glutamine starvation inhibits ERK phosphorylation but we do not know if this kinase or any of its pathway components is actually sensing

intracellular glutamine or if another sensor is actually feeding into the MAP kinase pathway.

We have shown that glutamine is rapidly taken up during activation but we do not know which transporters are dominant in T cells. By investigating transporter expression when T cells are starved of glutamine as compared to inhibition of key signaling pathways, we can determine which are most important and which signaling pathways may regulate their activity. We have found that like primary T cells, T cell lymphoma cells also require glutamine, but because they are no longer regulated through the TCR, glutamine may regulate these cells differently. Understanding the role of glutamine in rapidly dividing cells can have good therapeutic effects for diseases associated with metabolic dysfunction like HIV, cancer and liver disease. Lymphoma cells may require glutamine or one of its metabolites for survival. Enzymes responsible for glutamine catabolism may be key regulators of lymphoma cell survival. This has proven to be the case in some tumors in which investigators have found that tumor cells require asparagine while normal cells can synthesize their own. Asparaginase supplementation has often been used in combination with other cancer therapies to treat some forms of leukemia in order to starve and eventually kill the tumor cells (Richards 2006). By acquiring a better understanding of the regulation of T cell activation based on glutamine availability, may reveal novel targets for immunomodulatory and anti-leukemia therapy.

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