

ABSTRACT

Title of Document: T CELL ACTIVATION REQUIRES GLUCOSE RETENTION AND USE VIA MECHANISMS MODULATED BY MAPK SIGNALING

Aimée Joy Marko, Master of Science, 2010

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T lymphocytes play a critical role in a cell-mediated immune response. During activation, extracellular and intracellular signals alter T cell metabolism in order to meet the energetic and biometabolic needs of a proliferating, active cell, but control of these phenomena is not well defined. The idea that metabolism can be regulated directly via receptor-mediated signals is being investigated. The T cell receptor (TCR) and CD28 pathways were stimulated and signaling was modified using chemical inhibitors of MAP kinases. Metabolic changes were monitored using assays for glucose uptake, hexokinase activity, glycolysis, Krebs Cycle, and pentose phosphate pathway. Two MAPK pathways (ERK, JNK) appear to be determining factors because their inhibition downregulated metabolism. Inhibition of another MAPK pathway (p38) causes some downregulation of these activities but the effect is not as strong as with the ERK and JNK inhibitors, suggesting that the p38 pathway is less important in regulating glucose metabolism.

T CELL ACTIVATION REQUIRES GLUCOSE RETENTION AND USE VIA
MECHANISMS MODULATED BY MAPK SIGNALING

By

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Dedication

Thank you, Mom and Dad, for supporting me throughout my schooling, for encouraging me, for living lives filled with hard work so that I could have a better chance – Susan Jzyk Marko, Stephen George Marko.

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SUMMARY

T lymphocytes play a critical role in a cell-mediated immune response. During activation, extracellular and intracellular signals alter T cell metabolism in order to meet the energetic and biometabolic needs of a proliferating, active cell, but cellular control of these phenomena is not well defined. Previous theories suggested increased metabolite use and subsequent catabolic derepression allowed metabolism to perform at maximum levels, without regulation, and that increased metabolism was simply an end result of signaling. An alternative idea - that metabolism can be regulated directly via receptor-mediated signals - is being investigated. To define the role of carbohydrate flux in T cell activation, the T cell receptor (TCR) and CD28 pathways were stimulated using antibody-coated beads. At 24 hours, metabolic changes induced by these signals were monitored using assays for glucose uptake, hexokinase activity, glycolysis, Krebs Cycle, and pentose phosphate pathway. Results showed that activated T cells dramatically raised glucose flux through GLUT1, hexokinase, and glycolysis and pentose phosphate pathway, but not Krebs Cycle. Then, signaling through the TCR and CD28 pathways was modified using chemical inhibitors of MAP kinases. Metabolic changes induced by these altered signals were monitored. Two MAPK pathways (ERK, JNK) appear to be determining factors because their inhibition downregulated glucose uptake, hexokinase activity, glycolysis and Krebs Cycle. Inhibition of another MAPK pathway (p38) causes some downregulation of these activities but the effect is not as strong as with the ERK and JNK inhibitors, suggesting that the p38 pathway is less important in regulating glucose metabolism. It also appears that T cells actively restrict glucose entry into the Krebs Cycle, though

other nutrients may still feed through it. These phenomena could have clinical implications for anti-cancer and immune response-modulating drugs.

INTRODUCTION

T cells play an important role in immune responses by detecting antigens presented on cell surfaces and coordinating the actions and fates of other cells. T cells show dramatic changes in cellular activity upon stimulation, including a need for increased metabolism. Changes in nutrient availability and metabolism within T cells may have a greater influence on the outcome of an activation event than previously thought. Current studies on T cell activation are revisiting metabolic analyses performed in the last century, but now the focus is on integration of the metabolic signal with other signaling pathways in order to construct a more holistic model of T cell activation and energy induction. The emerging paradigm is one where metabolism plays as significant a role as conventional signaling does in modulating and directing the cell's immune response [Fig. 1]. The idea now is that increased metabolism is not merely an afterthought in response to increased energetic and biosynthetic needs, but could instead be a mechanism to sense the availability of nutrients, provide feedback to the cell that will allow and enhance activation, and push the cell to channel glucose into biosynthetic products rather than energy production alone.

Resting lymphocytes depend upon growth signals from cytokines and low-level TCR stimulation in order to maintain homeostasis, and they don't upregulate expression of glucose transporters or change their metabolism or activation state in response to high levels of extracellular glucose (Rathmell et al. 2000; Rathmell et al. 2001). Glucose metabolism in resting T cells is split equally between lactate production (glycolysis), oxidation to CO₂ for energy production (glycolysis + Krebs Cycle and pentose

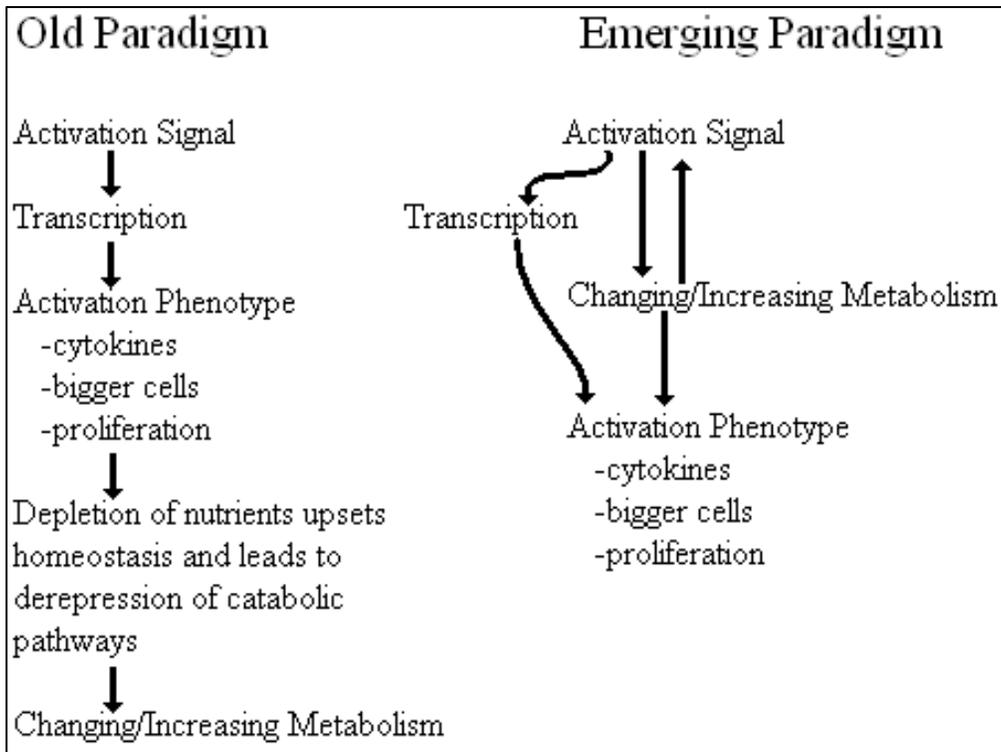


Figure 1. Paradigms of the role and regulation of metabolism during T cell activation.

Previous ideas held that the cell responds to a stimulation signal by increasing its uptake and metabolism. The proposed paradigm holds that the cell monitors both outside signals as well as available nutrients before it commits to activation. The work in this thesis supports the direct connection between the activation signal and changing/increasing metabolism.

phosphate pathway), and synthetic reactions (glycosylation reactions), while lactate production predominates in activated cells (Hume et al. 1978). These findings indicate that activation doesn't result in a straightforward upregulation of all metabolic pathways, and that simply having an abundance of glucose does not mean a cell will become activated; there must be some signal or combination of signals in addition to nutrient availability that changes the rate and fate of glucose carbon flux.

Lymphocyte activation leads to regulation of a change in metabolic state, but what the instructive signals are, and which cell components they change, are not well understood. Glucose metabolism in concanavalin A-activated T cells is skewed toward lactate production. In resting cells, 28% of glucose use can be traced to energy production and 38% to lactate production. In activated cells, the values are 20% and 58%, respectively (Hume et al. 1978). There are several different hypotheses why this altered state of metabolism in lymphocytes, termed aerobic glycolysis, happens. A switch to aerobic glycolysis could enable the cell to produce more energy while keeping electron transport chain activity, and thus free radical production, to a minimum (Brand and Hermfisse 1997). It could be a mechanism that allows the cell to shunt glucose toward anabolism rather than energy production. It could be an adaptation that allows T cells to become activated in a low-oxygen environment or it could be a correlate to the finding that oxygen can inhibit lymphocyte proliferation (Karlberg et al. 1981).

The change from resting to activated T cell begins with a signal through the T cell receptor (TCR). When the TCR is stimulated, signal transduction pathways are activated and transcription of gene products is enhanced. What those gene products will be is determined by concomitant signaling both within and from outside the cell. When signaling through only the TCR occurs, the cell enters a state called anergy which is characterized by an inability to respond to an antigen that would normally elicit an immune response. When signaling through the TCR occurs along with costimulation through CD28, the cell becomes activated and participates in an immune response. Some research has been done to determine how glucose fits into this activation model, and this thesis presents some questions and details some experiments which will help define the role of glucose in the immune response of lymphocytes.

The results derived from this thesis will be important not only in T cell research but in other areas of metabolism and lymphocyte research as well because B cells also upregulate glucose metabolism upon receptor stimulation (Doughty et al. 2006). It is important to study glucose metabolism in T cells to determine how it affects their function. It is meaningful to study metabolism in T cells because this knowledge can be incorporated into a broader model of general cell metabolism. Lymphocytes are a better model than more metabolically specialized cells like hepatocytes and adipocytes which primarily transport glucose in an insulin-dependent manner. Also, the metabolic difference between active and resting T cells is relatively large, which makes it easier to make statistically significant observations.

The long term goal of the proposed study is to increase understanding of what happens to the metabolism of lymphocytes during the time when they transform from resting to activated. Immunotherapies such as allergy injections, immunizations, and administration of IL-2 and other cytokines rely on providing an external signal to lymphocytes. The effects of glucose metabolism on cells could also be exploited in order to fine tune the complex mix of signals received by cells in order to achieve the desired immune response.

BACKGROUND

Glucose Transport

Glucose does not easily diffuse through membranes but must instead be transported across via a member of the hexose transporter family, so called because they transport the six-carbon sugars fructose, galactose and glucose. Through molecular cloning, a family of integral membrane glycoproteins containing at least 12 glucose transporter (GLUT) genes has been identified (Joost et al. 2002), many of which have high homology between mice and humans - *Glut1* from mice and its human homologue SCL2A1 are 96% similar. Most cells express more than one hexose transporter, and all are transmembrane proteins with 12 transmembrane domains, cytoplasmic C- and N-termini, an extracellular glycosylated loop between TM1-TM2, and an intracellular loop between TM6-TM7. Most glucose transporters work by facilitated diffusion; hexose transport is achieved by a series of conformational changes that move the hexose across the cell membrane from a binding site on one side to a binding site on the other side, the relative binding affinities of which differ. ATP is not used, though SGLUT1 and SGLUT5 are sodium-glucose symporters (Zhao et al. 2005). Some GLUTs can also catalyze the movement of glucose out of the cell by reversing the conformational changes that transported it in if the intracellular glucose concentration is high.

Glucose transporter 1 (GLUT1), the primary transporter found on lymphocytes (Rathmell et al. 2000), is found in various cell types throughout the body, and is

generally considered the ubiquitous vertebrate glucose transporter. It is also the cell surface receptor for Human T Cell Leukemia Virus (HTLV), and it can be blocked by HTLV-1 and -2 receptor-binding domains of the envelope glycoproteins (Manel et al. 2003). It can also be inhibited by cytochalasin B, dehydroascorbic acid (Agus et al. 1997), and a less specific inhibitor - phloretin (Chung et al. 2002). Its Michaelis-Menton constant (K_m) is 1-1.5mM which is slightly lower than the average blood glucose concentration of 4 - 8mM, so GLUT1 is saturated at physiological blood glucose levels, and glucose enters most cells at a fairly slow and steady pace through this transporter.

GLUT1 and GLUT3 (glucose/galactose) are similar, and are found in most cells. GLUT2 (glucose/galactose/fructose) is found in pancreatic beta cells and liver cells, and it transports glucose much more quickly than GLUT1. GLUT4 (glucose) is found in skeletal and cardiac muscle cells, as well as in adipocytes and hepatocytes, and surface expression of GLUT4 is upregulated by insulin receptor activation. GLUT5 (fructose) and SGLUT5 (glucose/galactose/sodium) are found on intestinal epithelium. The homeostatic effect of the various transporters is that cells take up glucose at a steady rate from the blood via the slower GLUTs, and when the concentration of glucose increases after a meal, GLUT4 reacts quickly to sequester the glucose into specialized cells.

Hexokinase

Hexokinase phosphorylates glucose into glucose-6-phosphate and allows it to enter a number of intracellular metabolic processes, such as pentose phosphate pathway or

glycolysis. It can catalyze the magnesium-dependent and ATP-dependent conversion of D-fructose, 5-keto-D-fructose, D-glucose, 2-deoxy-D-glucose, D-mannose and D-glucosamine to their respective hexose-6-phosphate forms. This phosphorylation transforms the glucose into an anion, trapping it in the cell because large anions cannot cross cell membranes and because cells do not have membrane transport systems for phosphorylated sugars. This conversion changes glucose from a biologically inert molecule into a reactive one that can enter metabolic pathways, and it also removes glucose from equilibrium which allows the cell to maintain a favorable concentration gradient. Mitochondrially-bound hexokinase has been shown to be the driving force for the “Warburg Effect” - extremely high glycolytic activity – that takes place aerobically in tumor cells and other activated cells (Bustamante and Pedersen, 1977). Hexokinase requires magnesium ions for its catalytic activity. It is activated by catecholamines and related compounds (Harrison *et al.* 1972). Calcium ions do not affect its enzymatic activity. It is inhibited by its product, glucose-6-phosphate.

Glycolysis

Glucose may enter the metabolism of the cell via glycolysis or via the pentose phosphate pathway (PPP), both of which take place in the cytoplasm. Glycolysis is the process whereby glucose is broken down into pyruvate [Fig. 2] which may then enter the Krebs Cycle, be metabolized into lactic acid and excreted, or enter other metabolic pathways. Each step of glycolysis either releases a small, capturable amount of energy from the glucose molecule, or rearranges the molecule so that

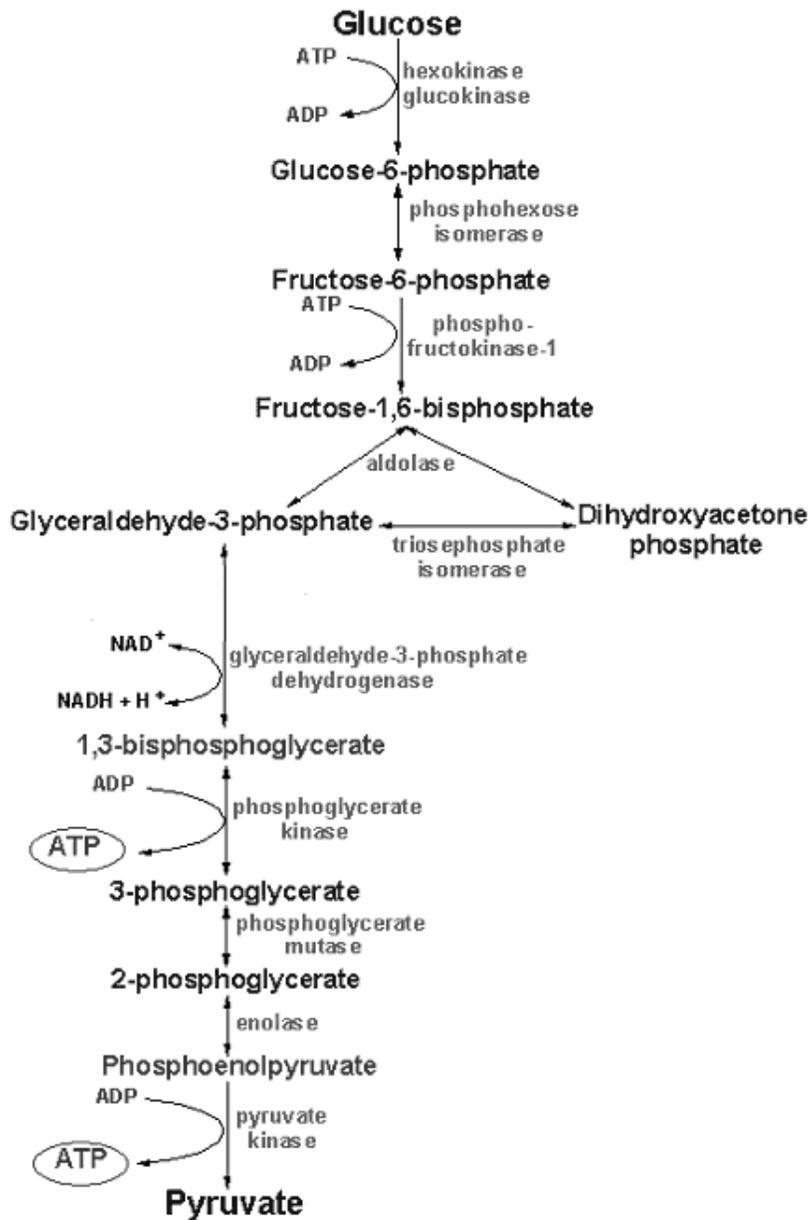


Figure 2. Glycolysis. This metabolic process uses enzymes to degrade glucose to pyruvate, creating ATP and recharging NADH. The first step, creation of glucose-6-phosphate, is mediated by hexokinase which is one of the enzymes whose activity is reported in this thesis. Production of water during the eighth reaction mediated by enolase is measured in the glycolysis assay.

energy may be captured during subsequent steps. All steps take place anaerobically, and this is the primary pathway a eukaryotic cell uses for energy production if there is no oxygen available. Glycolysis begins when glucose is phosphorylated into glucose-6-phosphate by hexokinase + ATP. The third step of glycolysis is the step that commits glucose to glycolysis: once phosphofructokinase, an allosterically regulated enzyme controlled by several inhibitors and activators, phosphorylates fructose-6-phosphate, the reverse reaction does not readily proceed spontaneously because of the high free energy requirement in the reverse direction ($\Delta G^{\circ} = +5.4\text{kcal/mol}$). This is also the rate-limiting step of conversion of glucose to pyruvate.

Aerobic Glycolysis

Aerobic glycolysis is the term used to identify upregulated glycolysis in the presence of oxygen without a corresponding consumption of oxygen. It is characterized by high glycolytic activity even in the presence of oxygen (contrary to the Pasteur effect), and inhibition of oxidative phosphorylation by high levels of exogenous glucose (Rodríguez-Enríquez et al. 2001). It has been shown that stimulation of lymphocytes with the plant lectin concanavalin A causes a proliferative response during which aerobic glycolysis occurs (Hume et al. 1978). Bauer et al. (2004) have shown a similar, dose-dependent hematopoietic stem cell response to the addition of IL-3 in an IL-3-dependent cell line.

Aerobic glycolysis may have a several-pronged influence on the cell. First, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been shown to reverse the

inhibition of cyclin B-cdk1 activity that is caused by SET protein. When cell lines are transfected with a GAPDH expression vector, the number of mitoses increase and cell cycle progression accelerates (Carujo et al. 2006). Thus, upregulation of glycolysis may help to induce cell proliferation by this method. Second, upregulation of glycolysis and concomitant downregulation of the Krebs Cycle may lower the amount of NADH and FADH₂ produced while keeping ATP production at a sufficient level to ensure cell growth. In this way, cells will have sufficient energy without excess production of free radicals made during electron transport chain synthesis of ATP at a time when delicate DNA is replicating in anticipation of cell cycle initiation (Brand and Hermfisse 1997). Third, it is thought that the major function of aerobic glycolysis is to maintain sufficient amounts of glycolytic intermediates to act as precursors for macromolecule synthesis during a time when the cell is rapidly turning-over old molecules and synthesizing new ones. Aerobic glycolysis is not simply a response to the need for ATP; inhibition of glycolysis in the presence of alternative Krebs Cycle metabolites blocks PHA-induced proliferation (Roos and Loos 1973). It is not known whether the effects of glycolysis upregulation are a direct or indirect cause of mitosis, but it is important to recognize the concurrence of these events because it adds support to the hypothesis that T cell activation (of which proliferation is a part) and metabolism work synergistically.

Krebs Cycle

Krebs Cycle oxidizes acetyl CoA into CO₂, phosphorylating energy (GTP), and reducing energy (FADH₂ and NADH) within the mitochondrial matrix [Fig. 3]. The

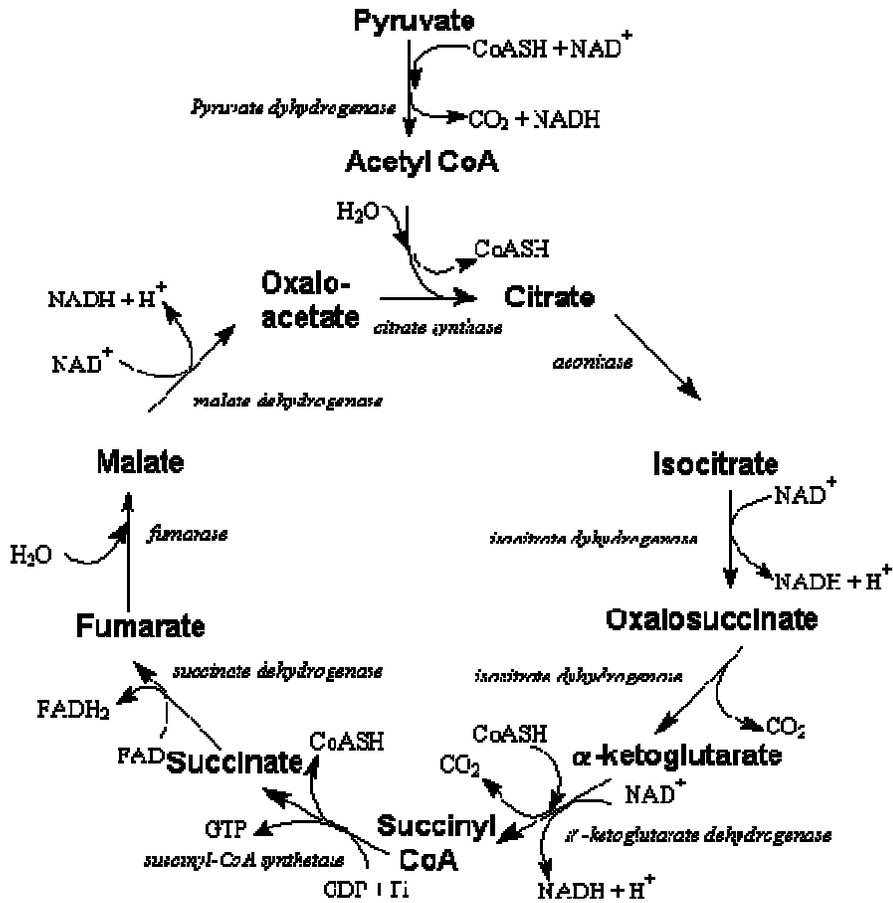


Figure 3. Krebs Cycle. This metabolic process rearranges the carbon backbone of pyruvate into various metabolites, releases CO_2 and recharges NADH , FADH_2 and GTP . Carbon from glucose gets rearranged and recycled through this pathway, and its main product is energy rather than biometabolites.

reducing energy may be used to generate ATP via oxidative phosphorylation in the electron transport chain (ETC) of molecules embedded in the mitochondrial cristae. Byproducts of ATP generation by the ETC include free radicals which may damage DNA, so the advantage of extracting maximum ATP by instructing glucose metabolites to pass through the Krebs Cycle may be offset by the deleterious effects of the free radicals on DNA and protein synthesis in a blasting T cell. Therefore, Krebs Cycle may be an important chain of events for a growing and differentiating T cell to control. Also, the metabolites of the Krebs Cycle are not constrained to the Cycle, but may be used as raw materials in many other pathways in the cell. One advantage to this is that the cell, by controlling the amount of particular Krebs Cycle proteins, may regulate which metabolites are in abundance at any one time so that the cell may shunt metabolites to other metabolic pathways in a more direct and useful manner.

Pentose Phosphate Pathway

The pentose phosphate pathway is crucial to growing and proliferating cells - in rapidly proliferating hybridoma cells, >90% of glucose enters the pentose phosphate pathway (Bonarius et al. 2000). Glucose-6-phosphate enters the PPP, the process whereby the carbon skeleton of glucose is used to synthesize the pentose sugar ribose-5-phosphate. A byproduct of this pathway is the phosphorylated sugar fructose-6-phosphate [Fig. 4]. In performing these reactions, the PPP also recharges a reducing equivalent by converting NADP^+ to NADPH. Ribose-5-phosphate and NADPH are crucial to cells which are growing and dividing; ribose-5-phosphate is used to make

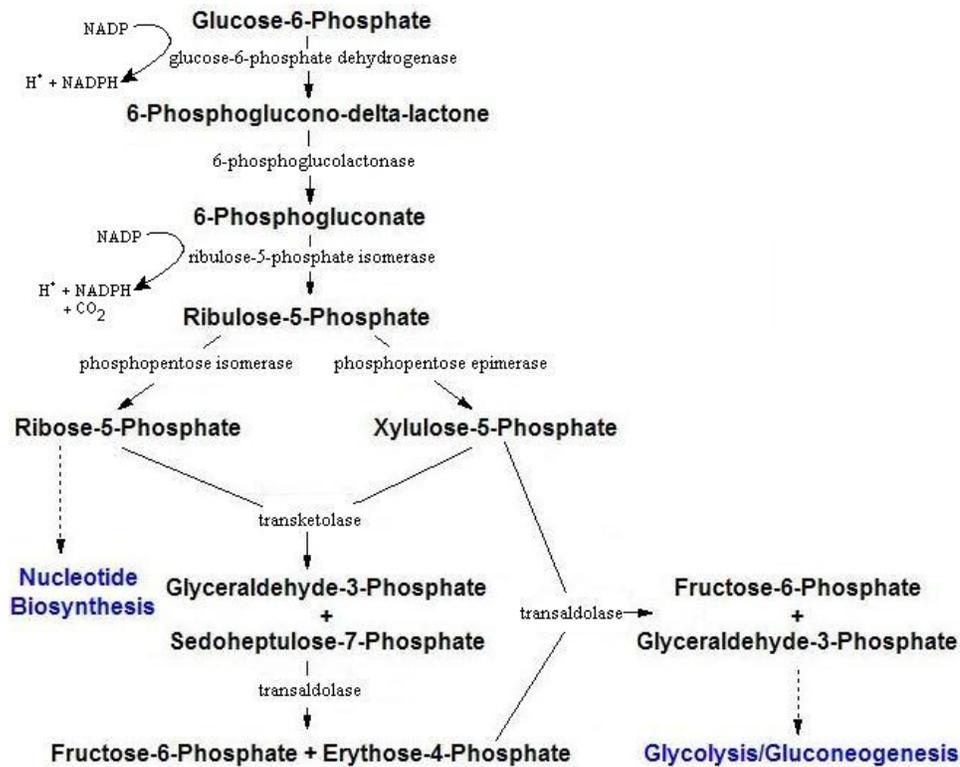


Figure 4. Pentose Phosphate Pathway. This metabolic process converts glucose-6-phosphate to fructose-6-phosphate, ribose-5-phosphate and other biometabolite products while recharging NADPH and releasing CO₂. The two main functions of this pathway are to provide the cell with reducing energy, in the form of NADPH, which can be used as energy to drive biosynthesis or as reducing energy to protect the cell from free radicals, and to create 4- and 5-carbon sugars which are precursors to amino acid and nucleotide synthesis.

nucleic acids while fructose-6-phosphate is a substrate in the glycolytic pathway, and NADPH is used for reducing power (an electron source) in anabolic reactions such as fatty acid and steroid biosynthesis, as well as in conversion of ribonucleotides to deoxyribonucleotides. In contrast, the NAD^+ used in glycolysis primarily functions as an electron acceptor for oxidative, catabolic reactions. PPP enzymes are found in high levels in liver, adipose, adrenal cortex, testicular, and lactating mammary gland tissues. In fact, 30% of oxidation in the liver is due to the PPP. There are two phases to the PPP, oxidative and nonoxidative. The reaction performed by glucose-6-phosphate dehydrogenase (G6PD) is the rate-limiting step in the PPP, as well as the step that commits glucose-6-phosphate to the PPP.

Properties of T Cell Activation

Activation can be induced by TCR stimulation and CD28 costimulation, concanavalin A, phytohaemagglutinin (PHA), and treatment with other mitogenic lectins. PHA activation can be inhibited by using 2-deoxyglucose (2-DOG) (Roos and Loos 1973). 2-DOG is a glucose analog that can be phosphorylated and trapped in the cell but cannot enter metabolism, and it kinetically inhibits the activity of phosphofructokinase, so being able to block activation with 2-DOG shows that glucose metabolism is a critical component of the process that leads to activation.

The hallmarks of T cell activation are proliferation and IL-2 production, but there are numerous ways to confirm activation in an experimental setting, such as by testing for metabolic end products or surface markers. Media color, cell size, viability and

morphology changes can also be quickly assessed to determine if in-vitro activation has been successful and further experiments can be run. During T cell activation, lactic acid is produced in excess and is excreted out of the cell. In vivo, the lactic acid is pumped into the blood and transported to the liver for further metabolism. In vitro, cells are typically cultured at ~pH7.2, and an increase in extracellular acidity can be detected with many commercial tissue culture media that contain phenol red, which turns from red to yellow at a pH of about 6.8. A direct assay for lactic acid can be done, but simply looking at the media color can reveal a successful activation. Activation causes upregulation of several surface markers like CD25 and CD44 that can be detected with fluorescent antibodies. Surface marker assays are an ideal check to determine if activation was successful because they can be run quickly and few cells are needed.

T Cell Activation Signals

Recognition of MHC/peptide by a TCR begins a cascade of signal transduction within the cell that ends by activating a set of transcription factors which regulate expression of various genes. Depending on the amounts and types of costimulation given during the period of TCR engagement, cells may become anergic or activated (Alegre et al. 2001). When the TCR and its associated molecules (ζ -chains, CD3 δ , - ϵ and - γ) and its accessory molecule (CD4 or -8), productively interact with an MHC/peptide complex, a cSMAC (central SupraMolecular Activation Cluster) is formed on the lipid raft with a pSMAC (peripheral SupraMolecular Activation Cluster) around it comprised of LFA-1 and the cytoskeletal protein, talin (Potter et al. 2001). These

complexes are the beginning of a series of events that lead to clustering and scaffolding of immune activation molecules.

Once an immunological synapse (IS) is formed, signaling molecules are in place to become activated. The SRC family kinase LCK, associated with CD4 or -8, phosphorylates tyrosine residues in the ITAMS of the TCR- ζ and CD3 molecules. ZAP70 now associates at the ITAMs via its SH2 domains and is itself phosphorylated. Two adapter proteins, LAT and SLP76, are phosphorylated by ZAP70, and they in turn recruit PLC gamma. Activated PLC cleaves the membrane lipid PIP2 into IP3 and DAG. IP3 increases intracellular Ca^{2+} by releasing stores from the endoplasmic reticulum, which causes opening of cell membrane Ca^{2+} channels. Ca^{2+} activates calcineurin, a ser/thr phosphatase, which cleaves several phosphates from the transcription factor NFAT. NFAT then translocates to the nucleus. DAG combines with Ca^{2+} to activate the ser/thr kinase PKC which activates CARMA1, bringing TAK1 and IKK into close proximity and allowing TAK1 to phosphorylate IKK. IKK adds two phosphates to I κ B which marks it for degradation by the ubiquitin pathway. Once freed from I κ B, the transcription factor NF κ B is allowed to translocate to the nucleus. SLP76 also activates the guanine nucleotide exchange factors SOS and VAV which then initiate the MAP kinase cascades $\text{SOS} \rightarrow \text{ERK}$ and $\text{VAV} \rightarrow \text{JNK}$. ERK and JNK translocate to the nucleus where they phosphorylate, respectively, the transcription factors ELK and JUN. JUN associates with FOS to form the transcription factor AP-1.

It has been shown that although upregulation of biosynthesis can be sustained over the short term, extracellular signals are necessary to sustain this upregulation in order to transform a resting cell into a productive, activated lymphocyte. One of these extracellular signals is activation of the costimulatory molecule CD28 by either B7.1 (CD80) or B7.2 (CD86), both members of the B7 family of ligands (Sharpe and Freeman 2002). There have been studies connecting immunoreceptor signaling to metabolic control: it has been shown that the CD28 activation pathway has some components in common with the insulin receptor/Glut4 activation pathway, notably AKT, which stimulates glucose transport and glycolysis (Parry et al. 1997) and promotes resistance to cell death-by-neglect (Rathmell et al. 2003), though the specific targets of AKT integral to T cell activation have not been defined (Frauwirth and Thompson 2004). Other systems have shown that upregulated levels of AKT are associated with increased expression and cell surface localization of Glut1 (Rathmell et al. 2003) and that AKT is involved in the insulin/Glut4 and Glut1 regulation of glucose metabolism (Hernandez and Lorenzo 2001).

Metabolic Control and Lymphocyte Function

In microorganisms, the presence of extracellular nutrients results in cell growth and proliferation, but in metazoans, cell growth and proliferation are controlled by external signals. Metabolism of lymphocytes is limited by trophic signals, low-level growth signals from cytokines or surface molecules on other cells, not nutrient availability. Without extracellular signals, T cells reduce glucose import to levels below those necessary to maintain homeostasis (Rathmell et al. 2001).

Phosphoinositide-3-kinase (PI3K) and Protein kinase B (AKT) activity, both mediated by signaling receptors, are necessary for upregulation of glucose metabolism. PI3K activates AKT, but in the absence of PI3K activity, constitutively active AKT will cause upregulation of glucose metabolism (Frauwirth et al. 2002). Metabolism can, in turn, control activation state. Glucose deprivation inhibits high production of IFN γ , a trait of CD8⁺ effector T cells, but not IL-2 production, a trait of both undifferentiated and effector cells (Cham and Gajewski 2005). Blockade of glycolysis with 2-deoxyglucose inhibits PHA-mediated activation (Roos and Loos 1973). Lymphocytes deprived of nutrients become atrophied and exhibit downregulated *glut1* and a reduction in ATP and mitochondrial potential, which suggests that atrophy results from depletion of metabolic substrates (Rathmell et al. 2000). Examined from another perspective, kinetic inhibition of glycolysis via prevention of lactate transportation inhibits lymphocyte activation. Without monocarboxylate transporter function, aerobic glycolysis cannot proceed because of accumulation of lactate and protons inside the cell (Murray et al. 2005).

These studies show that metabolism, nutrient availability and activation are intimately intertwined. Our research will examine how glucose metabolism can be regulated directly via receptor-mediated signals.

EXPERIMENTAL PROCEDURES

Antibodies and reagents

Anti-CD3 (mAb 145-2C11), anti-CD28 (mAb 37.51), control hamster IgG, and PE-labeled anti-Thy1.2, antibodies were purchased from eBioscience (San Diego, CA). Anti-PLC γ and anti-phospho-PLC γ (Y783) antibodies were from Cell Signaling Technology (Danvers, MA). HRP-conjugated anti-mouse IgG and anti-rabbit IgG were from Bio-Rad (Richmond, CA). ERK inhibitor (U0126) and JNK inhibitor (SP600125) and p38 inhibitor (SB203580) were purchased from BIOMOL International, L.P, (Plymouth Meeting, PA).

Animals

C57BL/6J mice (6-8 weeks old) were purchased from The Jackson laboratory (Bar Harbor, ME). All mice were maintained in ventilated M.I.C.E. microisolator cages (Animal Care Systems, Littleton, CO) in the University of Maryland animal facility (College Park, MD). Animals received humane care in compliance with the National Institutes of Health "Guide for the Care and Use of laboratory Animals".

T cell purification

For uptake, glycolysis, FLOW, Thymidine Incorporation and Western assays, murine T cells were purified from lymph nodes and spleens using the Dynal Mouse T Cell Negative Isolation Kit (Invitrogen Corporation, Carlsbad, CA, USA) according to manufacturer's protocol and as previously described. Purified T cells were >95% Thy1 positive, as determined by flow cytometry.

Cell culture

All cells were maintained in RPMI1640 medium (Mediatech, Herndon, VA) supplemented with 10% FBS (Hyclone, Logan UT), 200 μ M glutamine, penicillin/streptomycin, 10mM HEPES buffer, MEM non-essential amino acids, and 55 μ M 2-mercaptoethanol at 37 degrees Celsius in a 5% CO₂ atmosphere.

For all experiments, T cells from C57BL/6 mouse spleens and lymph nodes were enriched using a negative purification procedure, then stimulated for 24 hours in complete RPMI culture media using beads to provide stimulated and nonstimulated conditions. Cells were harvested at 24 hours, mixed with Trypan Blue and counted to normalize live-cell concentrations.

Antibody-coated beads

Dynalbeads M-450 Tosylactivated were prepared according to manufacturer's instructions. Beads were coated with 75µg/mL anti-CD3e and 75µg/mL anti-CD28 (stimulating condition) or 75µg/mL anti-Syrian hamster IgG and 75µg/mL anti-Armenian hamster IgG (75µL from a 1mg/mL stock). Beads were washed after incubating to adhere the antibodies, then stored in buffer with 0.02% sodium azide. Beads were washed twice with PBS before any cell culture experiments to rid them of the sodium azide. Final concentration of beads is 4×10^7 beads/mL, and beads are used at 3 beads per cell during stimulations.

Proliferation assay

Proliferation after three days after stimulation in vitro was determined by [³H] thymidine incorporation. Cells were pulsed for 6-8h with 1 µCi/well of [³H] thymidine (MP Biomedicals, Solon, OH), transferred to glass fiber filters with a 96-well cell harvester (Tomtec, Hamden, CT), and analyzed by liquid scintillation using a 1450 Microbeta Trilux scintillation counter (Wallac, Turku, Finland).

T cell stimulation

T cells were stimulated with anti-CD3 and anti-CD28 antibody-coated beads. Briefly, 1×10^6 cells/mL samples were incubated with anti-CD3, anti-CD28 beads, at a ratio of 3 beads per cell, in complete RPMI1640 medium at 37 degrees Celsius for 24 hours. For Krebs Cycle and pentose phosphate pathway experiments only, 10×10^6 cells/mL samples were used. For the MAP kinase inhibition assays, cells were incubated with 2.6 μ M ERK inhibitor (U0126), or 50 μ M JNK inhibitor (SP600125), or 20 μ M p38 inhibitor (SB203580) along with anti-CD3 and anti-CD28 antibody-coated beads. The inhibitors were also added to the complete RPMI medium during glucose uptake and glycolysis assay incubations. The concentrations used for these pharmacological inhibitors were obtained by titration experiments to look for functional inhibition (JNK inhibitor, ERK inhibitor, data not shown) or from established protocols (p38 inhibitor, ERK inhibitor). All inhibitors were dissolved at 1000x in DMSO and mixed into the reaction wells with cells prior to addition of stimulating beads.

Western blots

The T cells were stimulated as above and lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10mM NaF, 1mM Na_3VO_4 in PBS) The lysates were resolved on a 12% SDS PAGE gel ($\sim 1.25 \times 10^6$ cells/sample) and transferred onto nitrocellulose membrane. The blots were blocked with 5% non-fat dry milk in PBS/0.1% Tween-20 (PBS-T) and probed with primary antibody (1:1000 in PBS-T)

followed by HRP conjugated anti rabbit and anti mouse IgG respectively. The bands were visualized with SuperSignal West Pico Chemiluminescent substrate (Pierce).

Beads for Stimulating and Nonstimulating Conditions

For all experiments, T cells from C57BL/6 mouse spleens and lymph nodes were enriched using a negative purification procedure, then stimulating for 24 hours in RPMI culture media using beads to provide stimulated and nonstimulated conditions. Dynalbeads® M-450 Tosylactivated were (a) coated with 7.5µg/mL hamster anti-mouse CD3e and 7.5µg/mL anti-mouse CD28 ("stimulated" condition) or (b) coated with isotype-equivalent 7.5µg/mL Armenian hamster anti-TNP IgG and 7.5µg/mL Syrian hamster anti-TNP IgG ("nonstimulated" condition).

Glucose Uptake Assay

The rate of glucose uptake was measured by assaying uptake of radioactive 1,2[³H]-2-deoxyglucose (2-DOG) – a glucose analog that is not degraded – in stimulated and nonstimulated T cells in triplicate [Fig. 5]. Briefly, 3x10⁶ cells (1x10⁶ cells for each sample in the triplicate) were washed with PBS, incubated for 15 minutes at 37 degrees Celsius in glucose-free RPMI to clear the cytoplasm of glucose, then incubated for 10 minutes in glucose-free RPMI plus radiolabeled 2-DOG. 500µL microcentrifuge tubes were prepared by aliquotting 25µL perchloric acid plus 10%

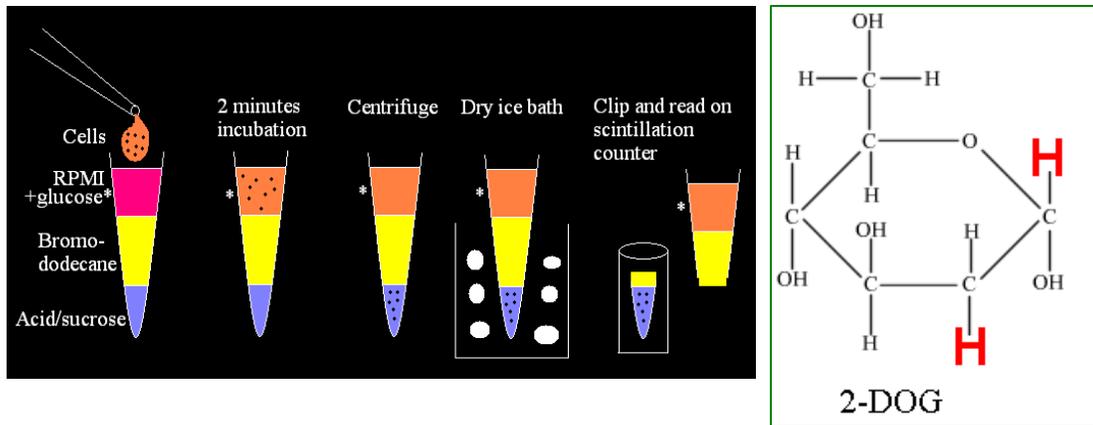


Figure 5. Glucose uptake assay illustration. Acid sucrose, bromododecane and RPMI with radioactive 2-DOG (inset) are layered in a centrifuge tube. Cells are added and allowed to take up 2-DOG, then are spun into the acid sucrose to stop the reaction. The whole tube is frozen in a dry ice bath and the cell fraction is clipped off and assayed for radioactivity.

sucrose in each tube, then layering 200 μ L bromododecane on top. An aliquot of cells in the 2-DOG mixture was carefully loaded on top of the bromododecane. The no-cell control was prepared with 9×10^6 beads (3×10^6 beads for each sample in the triplicate).

Tubes were spun in a microcentrifuge for 1 minute to pellet cells in the lower acid-sucrose layer. Tubes were placed in a solid CO₂/ethanol bath to freeze solid, then the acid-sucrose layer was clipped off into a scintillation tube and relative glucose uptake was determined via scintillation counter.

Hexokinase Assay

The hexokinase assay is based upon the reduction of NADP⁺ through a coupled reaction with glucose-6-phosphate dehydrogenase and is measured spectrophotometrically by detecting the rate of increase in absorbance at 340nm.. D-glucose + ATP in the presence of hexokinase yields glucose-6-phosphate + ADP. Then glucose-6-phosphate + NADP in the presence of glucose-6-phosphate dehydrogenase (G6PDH) yields gluconate-6-phosphate + NADPH. NADPH absorbs light at 340nm while NADP does not. Assay mix was made with 5.45g tris base, 7.4mL 1M MgCl₂, 0.666g glucose and adjusted to pH 8.5 with concentrated HCl. Per 100mL assay mix, 0.4mL 2.78 M thioglycerol (stored at -20 degrees Celsius) was added to each bottle immediately before use. Live T cells were counted and 10×10^6 cells aliquoted into microfuge tubes, centrifuged at 8000 rpm for 2 minutes, and the supernatant was aspirated. Cells were lysed in 0.01% Triton in PBS on ice for 15

minutes, then spun at 13,000 rpm at 4 degrees Celsius for 15 minutes, then lysate was moved to a new tube. The reaction mix was made with 90 μ L Assay Mix + thioglycerol, 1 μ L NADP (50mg/mL in 0.1M NaPhos, pH 7.0), 1 μ L G6PDH (100 units/mL in 0.02 M Tris with 0.2% BSA, pH 7.5), 3 μ L ATP (220 mM, pH 7.0). Assay mix and 5 μ L of each lysate were combined in UV transparent plates as quickly as possible prior to putting onto plate reader. The rate of change of OD at 340nm was measured using the basic kinetic protocol on the optical plate reader at room temperature every 30 seconds for 10 minutes. The change in absorbance per minute was determined only from the initial, linear portion of the curve. The initial slope was calculated using a linear best-fit line on a Microsoft Excel spreadsheet.

Glycolysis Assay

This assay measures glycolysis rate by detecting the generation of tritiated H₂O from 5-[³H]-glucose during the dehydration reaction mediated by enolase [Fig. 6]. The tritiated water is separated from the remaining radioactive glucose by a diffusion step, and the rate is calculated from the ratio of diffused to undiffused radioactivity. Fresh incomplete glucose-free RPMI was made, containing Q, P/S, Hepes, Amino Acids, and BME. Serum was not added. Live T cells were counted, then 1x10⁶ collected and washed with PBS, incubated for 15 minutes at 37 degrees Celsius in 0.5mL glucose-free RPMI to clear the cytoplasm of glucose. Make up 0.5 mL glycolysis reaction per sample + 0.5mL for negative control. 1M glucose (dissolved in RPMI) was added to glucose-free incomplete RPMI to a final concentration of 10mM

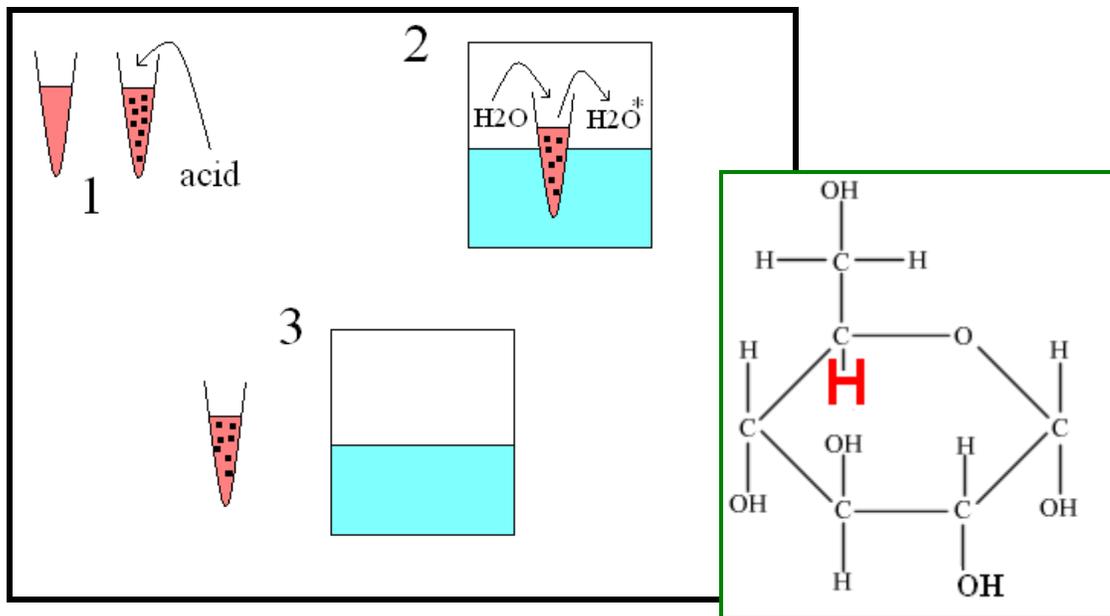


Figure 6. Glycolysis assay illustration. Cells are incubated with radioactive glucose (inset) for one hour, then reaction is stopped by the addition of acid. In a closed scintillation vial, radioactive water diffuses into an aliquot of distilled water while radioactive glucose remains inside the smaller tube. After 2 days, the reaction tube and the aliquot of distilled water are read separately on a scintillation counter and production of radioactive water via glycolysis is measured.

(25mM if DMEM was used to culture T cells), then just before the end of the 15 minute preincubation, 20 μ Ci (20 μ l of 1mCi/mL stock) of radioactive glucose was added per mL of diluted glucose-Krebs (10 μ l/sample). Cells were pelleted and supernatant decanted, then cells were resuspended in 0.5mL of the radioactive glucose-RPMI mix. Cells were transferred to a 24-well plate and incubated at 37 degrees Celsius in a 5% CO₂ incubator for exactly 1 hour. During the incubations, measurement tubes were set up. Each sample was measured in triplicate, using 3 PCR tubes per sample, including 3 tubes for the no-cell control and 3 tubes for the radioactive water control. 50 μ L of 0.2N HCl was added to each PCR tube. Per triplicate, 0.5mL non-radioactive distilled H₂O was added to each of 3 scintillation vials. The 1 hour incubation was stopped by pipetting cells to resuspend them, then aliquoting 50 μ L into each of three HCl-filled PCR tubes, for a final volume of 100 μ L per PCR tube. The diffusion control was run by adding 1 μ Ci (1 μ L of 1mCi/mL stock) of ³H₂O to 200 μ L of incomplete RPMI/10mM glucose mix, then aliquoting 50 μ L into triplicate PCR tubes filled with 50 μ L 0.2N HCl. The PCR tubes were transferred into the scintillation vials, capped tightly, and sealed with parafilm. Diffusion to occur at room temperature until equilibrium was established, approximately 2 days. A second set of scintillation vials was set up, parallel with the first. The PCR tubes were transferred to the new scintillation vials, then 0.5mL H₂O was added to each vial. To adjust pH, 100 μ L 0.1N HCl was added to each of the original diffusion volumes. 3mL Cytoscint was added to all tubes, mixed thoroughly, and counted on a scintillation counter.

Calculating the glycolytic rate

- 1.) Determine the diffusion fraction ($\text{frac}\{\text{diff}\}$). Divide the diffused $^3\text{H}_2\text{O}$ counts by undiffused counts for each triplicate of the diffusion control and average the triplicates.
- 2.) Determine the background diffusion rate ($\text{frac}\{\text{bkgd}\}$). Divide the diffused counts by undiffused counts for each triplicate of the cell-free control and average.
- 3.) Calculate the sample diffusion ratios ($\text{sample}\{\text{diff}\}$; diffused counts/undiffused counts) for each experimental triplicate set.
- 4.) The glycolytic rate, in nanomoles glucose per million cells per hour is: $[\text{sample}\{\text{diff}\} - \text{frac}\{\text{bkgd}\}]$ divided by $[\text{frac}\{\text{diff}\}] \times 5000$ nmol glucose if you used 10mM glucose in RPMI, or $\times 12500$ nmol glucose if you used 25mM glucose in DMEM.

Pentose Phosphate Pathway and Krebs Cycle Assays

Krebs Cycle and pentose phosphate pathway activities were measured using ^{14}C -glucose assays (Hume et al. 1978) by detecting release of radioactive carbon dioxide from D-[1- ^{14}C]-glucose or D-6- ^{14}C]-glucose [Fig. 7]. Fresh complete glucose-free RPMI was made, containing Q, P/S, HEPES, Amino Acids, BME and 10% dialyzed, heat-inactivated FBS. GF/B Whatman filter paper circles were cut in half, rolled, and placed in Kontes Center wells. The stem of the center well was pushed through a Kontes stopper, leaving a 1cm space between the well and the stopper. Live T cells

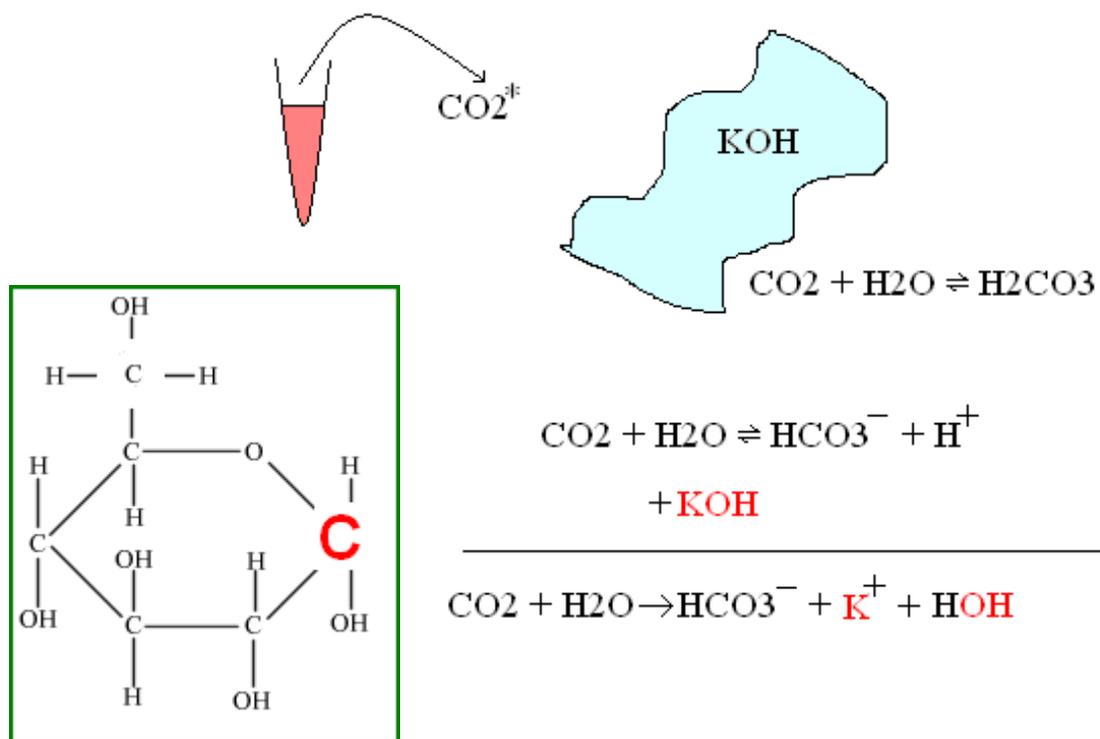
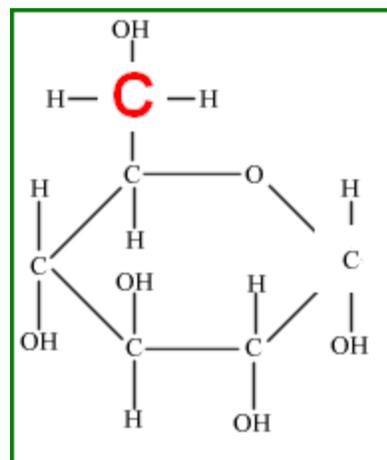


Figure 7. Krebs Cycle and Pentose Phosphate Pathway assay illustration. In a closed system, glucose that is radioactive on the 1 carbon (inset above) is mixed in RPMI and cells are allowed to metabolize it for 8 hours. Radioactive carbon dioxide is given off in both Krebs Cycle and PPP, and can be captured on a tissue soaked with KOH. The tissue is then assayed for radioactivity. A separate reaction is performed with glucose that is radioactive on the 6 carbon (right), which is released as radioactive carbon dioxide only in Krebs Cycle. The amount of CO_2 given off in the Krebs Cycle and PPP can be calculated using the ratio of the two separate assays.



were counted and 5×10^6 cells aliquoted into microfuge tubes, centrifuged at 8000 rpm for 2 minutes, and the supernatant was aspirated. Cells were washed in 1mL warm (37 degrees Celsius) complete glucose-free RPMI, centrifuged, the liquid aspirated, then finally resuspended in 0.5mL complete glucose-free RPMI plus 10% dialyzed FBS. Cells were allowed to rest 15 minutes at 37 degrees Celsius to deplete internal glucose stores. Two reaction solutions were made in complete glucose-free RPMI + 10% FBS, one containing C1-glucose and one containing C6-glucose, both at a concentration of $1.0 \mu\text{Ci/mL}$. 0.5mL of a radioactive solution was added to each 0.5mL aliquot of T cells, for a final concentration of $0.5 \mu\text{Ci/mL}$ and 5×10^6 T cells per mL. Fresh 5% KOH (0.05g/1mL) was made using 1-2 beads of KOH and distilled water. 100 μL KOH was added to the filter paper immediately before placing the stopper onto the vial. Vials were wrapped in parafilm and incubated for 8 hours at 37 degrees Celsius. At 8 hours, filter papers were removed to scintillation vials containing 2mL Cytoscint, then read on a scintillation counter.

RESULTS

Upregulated and Directed Glucose Metabolism Occurs During T Cell Activation.

It has previously been shown that stimulation increases glucose uptake and glycolysis in human T cells (Frauwirth et al 2000). In order to take advantage of the many genetic tools available in the murine system, C57BL/6 mice were chosen for this study. A survey of glucose metabolism has been performed in concanavalin A stimulated cells (Hume et al. 1978), but lectins work by nonspecific cross-linking of activation molecules. This model uses beads coated with anti-CD3e and anti-CD28 antibodies in order to more specifically stimulate changes in glucose metabolism. The magnetic polystyrene antibody-coated beads are 4.5 μ m in diameter, mimicking cell shape and size, and so may offer a better method of stimulation than free antibodies + crosslinker antibodies, or antibody-coated plates. This method is also useful to limit the effect of Antigen Presenting Cells on T cells - signals from cytokines are known to affect metabolism, and stimulation from APCs may affect different activation pathways.

To define glucose metabolism in murine T cells, several metabolic events were monitored: glucose uptake, hexokinase activity, glycolysis, Krebs Cycle, and pentose phosphate pathway activities. Glucose uptake is the first step in glucose metabolism, and its activity regulates the amount of glucose available to downstream metabolic pathways. Hexokinase activity traps glucose in the cell. Glycolysis and

Krebs Cycle activity provide energy to the cell. The pentose phosphate pathway provides reducing energy as well as metabolites used in cell structures.

To assay glucose uptake, glucose transfer across the cell membrane mediated by GLUT1 was monitored by measuring the accumulation of radioactivity of 2-DOG treated cells [Fig. 5]. Stimulated T cells show an upregulated rate of glucose uptake [Fig. 8]. Hume et al. found that lectin-stimulated lymphocytes deplete glucose from the medium twice as quickly as unstimulated cells do, which correlates with our findings.

To assay glycolysis, glucose flux through this pathway was monitored by measuring generation and diffusion of radioactive water $^3\text{H}_2\text{O}$ from metabolism of D-[5-(N)- ^3H]-glucose during the dehydration reaction mediated by enolase [Fig. 6]. Stimulated T cells showed a dramatically upregulated rate of glycolysis [Fig.9] measured in nanomoles of glucose per million cells per hour.

The strong induction of glucose flux through uptake and glycolysis was confirmed. With baseline measurements set, we then began inhibiting signaling pathways to determine if their function was necessary to upregulate glucose metabolism.

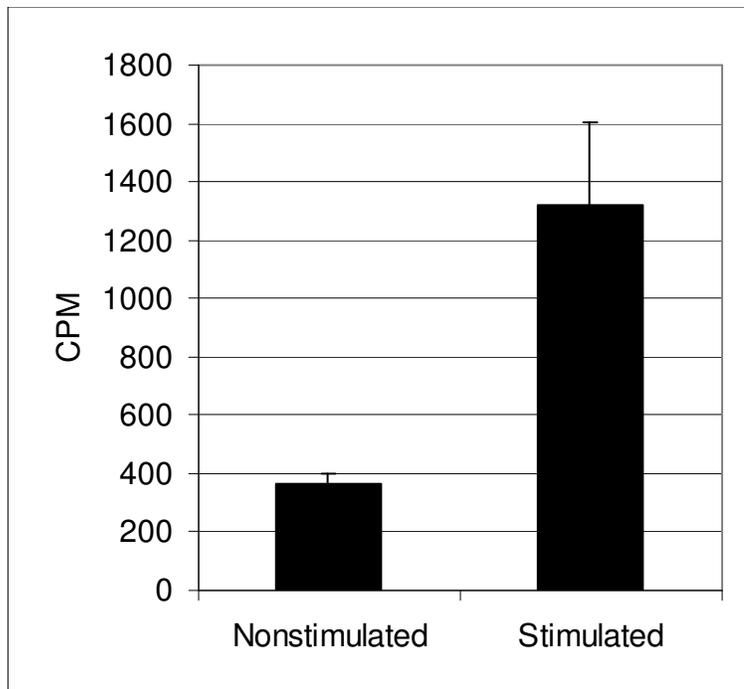


Figure 8. CD3/CD28 stimulation induces glucose uptake. Splenic T cells were stimulated for 24 hours in full-glucose RPMI with IgG or anti-CD3/anti-CD28 coated beads. Cells were counted and normalized to one million cells per trial. Three trials were allowed to take up tritiated glucose for 2 minutes in glucose-free RPMI. Uptake was measured via scintillation counting. Error bars represent one standard deviation.

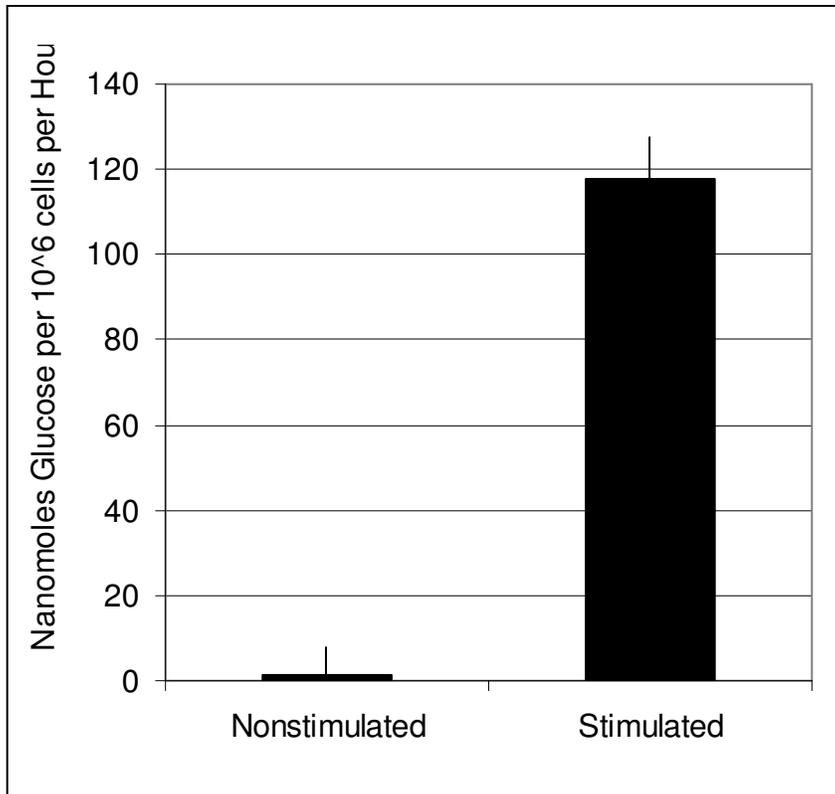


Figure 9. CD3/CD28 stimulation strongly induces glycolysis. Splenic T cells were stimulated for 24 hours with IgG or anti-CD3/anti-CD28 coated beads, and glycolysis rates were calculated by enolase assay as determined by release of the radioactive hydrogen on C-5 as water, measured via scintillation counting.

Glucose Metabolism Responds Strongly to Signaling through the MAP Kinase ERK and JNK Pathways, and to a Lesser Extent through the p38 Pathway.

T cell stimulation not only causes an upregulation in metabolism, but also sets into motion a host of other cell responses such as gene transcription and cell cycle induction. An important component of T cell signaling is activation of the MAP kinase family members ERK, JNK and p38. These signals were chosen because they are known to be activated by TCR stimulation. Glucose metabolism has been shown to rise when T cells are stimulated, and signaling pathways important to glucose metabolism need to be identified.

To determine whether MAP kinase signaling contributed to control of glucose metabolism, MAP kinase inhibitors were added during the stimulation incubation period, and metabolic events were monitored. C57BL/6 splenic and lymph node T cells were stimulated as before, with CD3/CD28 beads or IgG beads, +/- inhibitors, then cells were collected, washed and counted using Trypan Blue to normalize numbers of live cells per sample. It was determined that the concentrations of inhibitors did not cause large amounts of cell death at 24 hours [Fig. 10] so differences in measurements are true reflections of differing levels of metabolism, and are not due to lack of viable cells.

Inhibition of MAP kinases lowered glucose uptake [Fig. 11]. The ERK kinase inhibitor U0126 and the JNK inhibitor SP600125 lowered uptake almost to resting

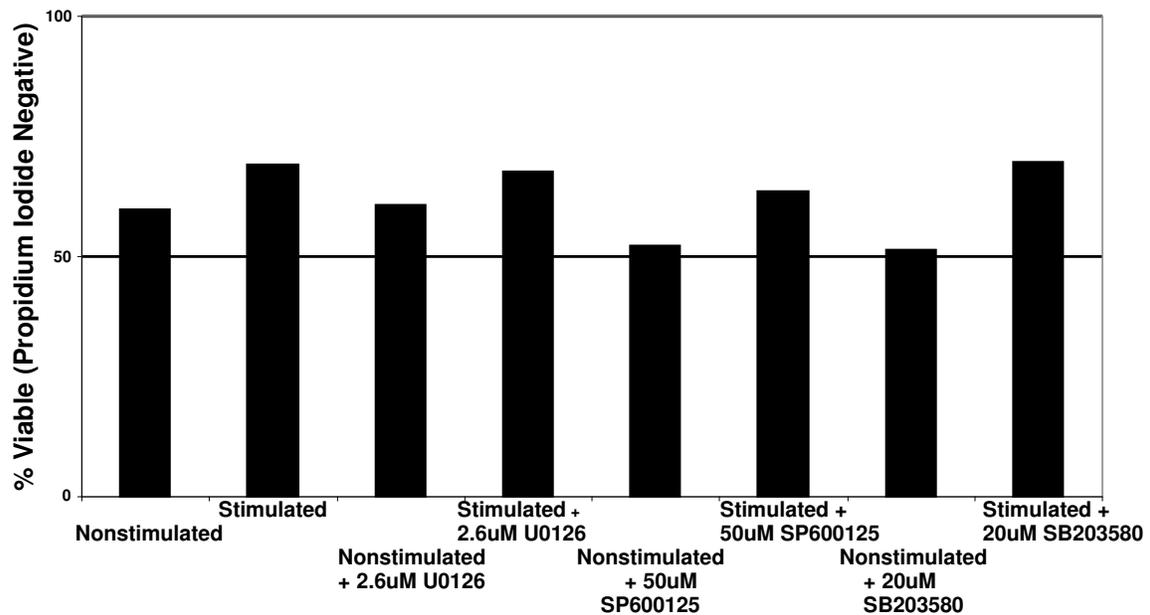


Figure 10. MAP Kinase inhibitors provide viable cells at working concentrations. A propidium iodide assay was used to determine that inhibitor concentrations did not affect cell viability at 24 hours. Splenic T cells were stimulated for 24 hours in the following conditions: IgG beads (Nonstimulated), anti-CD3/anti-CD28 beads (Stimulated), Stimulated + the recommended micromolar concentration of each inhibitor: 2.6 μ M ERK inhibitor (U0126), or 50 μ M JNK inhibitor (SP600125), or 20 μ M p38 inhibitor (SB203580). PI uptake was determined on FACS after a 10 minute pulse at room temperature. One trial was run for each condition and stopped when 20,000 cells had been assayed. Because some cell death in culture is normal at 24 hours, all metabolic assays were normalized to count living cells only.

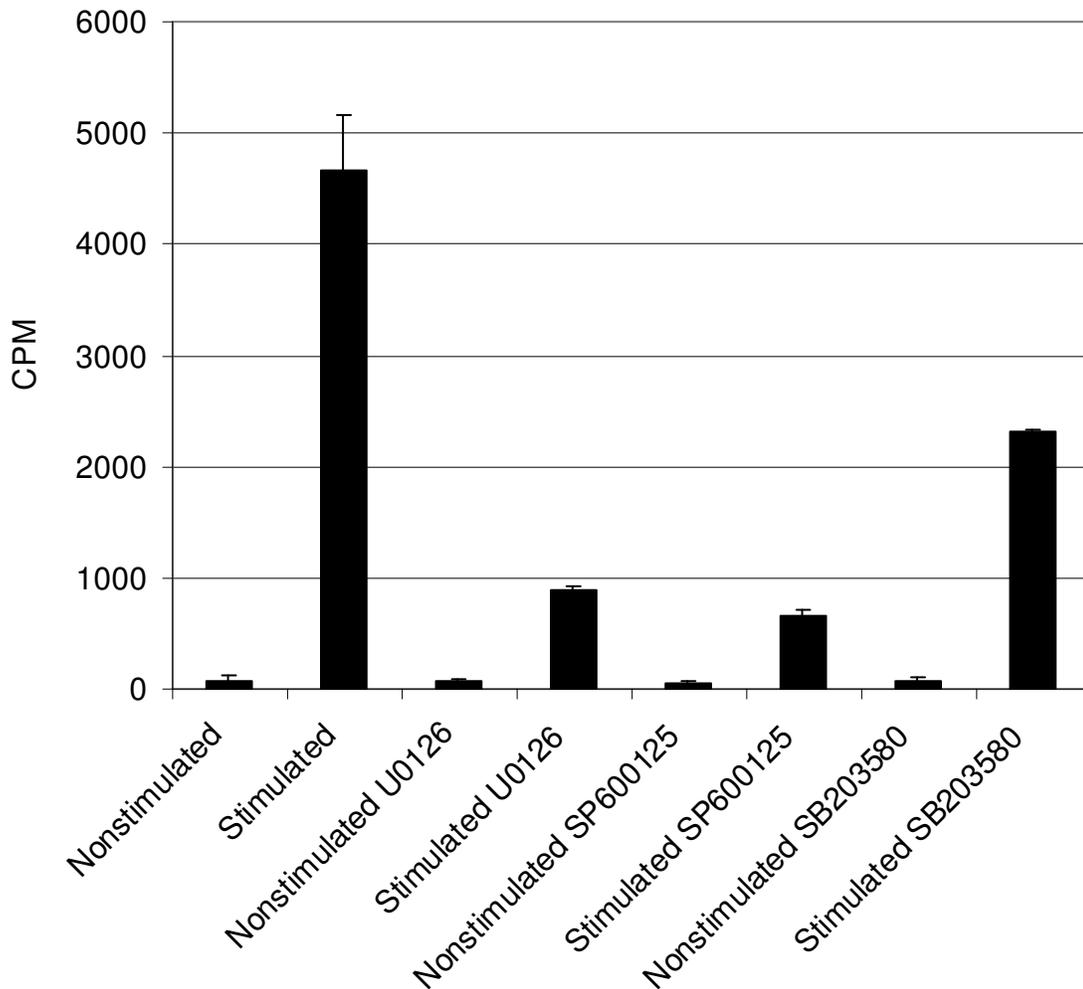


Figure 11. Inhibition of ERK, JNK, and p38 pathways lowers glucose uptake. Splenic T cells were stimulated for 24 hours in full-glucose RPMI with IgG or anti-CD3/anti-CD28 coated beads, +/- inhibitors: 2.6 μ M ERK inhibitor (U0126), or 50 μ M JNK inhibitor (SP600125), or 20 μ M p38 inhibitor (SB203580). They were then allowed to take up tritiated glucose for 10 minutes in glucose-free RPMI. Uptake was measured via scintillation counting.

levels, while the p38 inhibitor SB203580 lowered uptake to a lesser extent. Similar results were seen when glycolysis [Figs. 12 A and B] was measured.

With confirmation that MAP Kinase signaling played a direct role in upregulating glucose uptake and glycolysis, we then decided to follow glucose through downstream metabolic pathways in order to better define the nutritional value imparted by glucose during the dynamic process of activation.

Preliminary Results Indicate That Glucose Flux through Hexokinase, Krebs Cycle and Pentose Phosphate Pathways is Upregulated During T Cell Activation.

Unpublished experiments from our lab have shown that murine T cells stimulated in the absence of glucose fail to attain an activation state [Fig. 13]. That the activation of T cells requires glucose, and in fact glucose flow through uptake and glycolysis is upregulated during this event, indicates that this sugar plays a crucial role in providing energy to the cell or providing biometabolites for structures, or both. **To better define the role of glucose during T cell activation, we chose to examine three important downstream processes: hexokinase activity, Krebs Cycle and pentose phosphate pathway activity.**

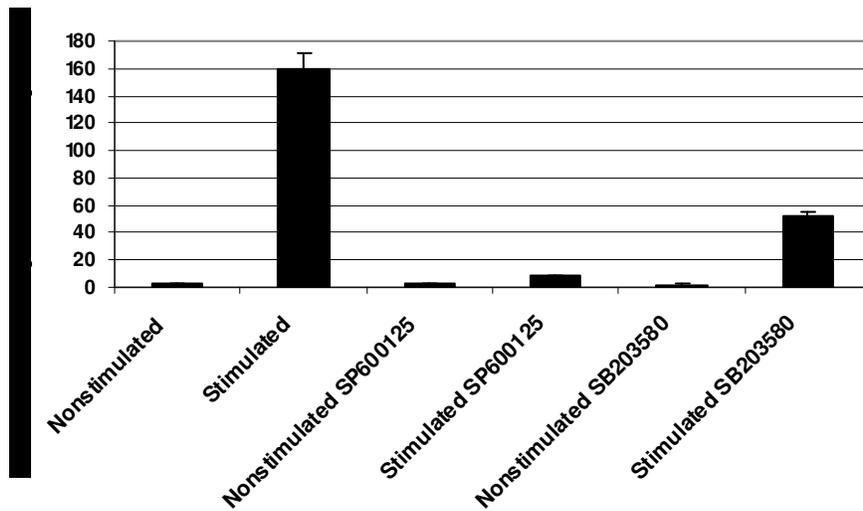
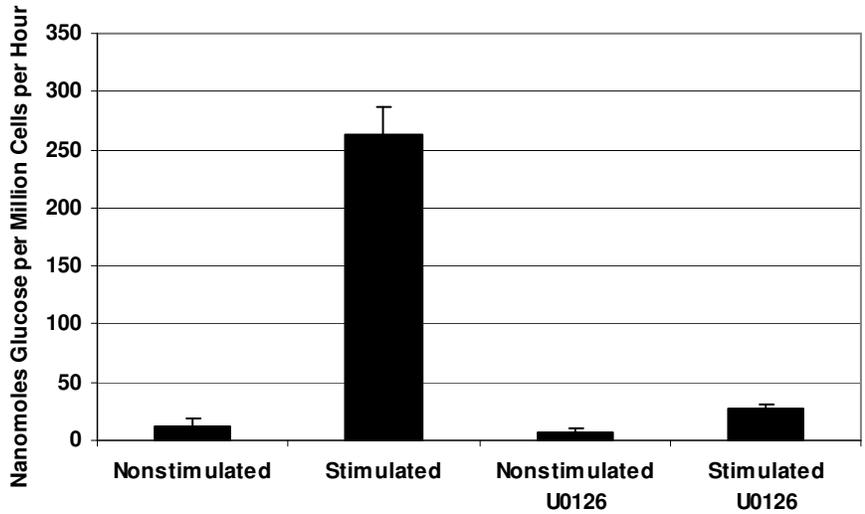


Figure 12. Inhibition of ERK, JNK and p38 pathways lowers glycolysis. Splenic T cells were stimulated for 24 hours in full-glucose RPMI with IgG or anti-CD3/anti-CD28 coated beads, +/- 2.6 μ M U0126 (A) or +/- 50 μ M JNK inhibitor (SP600125), or 20 μ M p38 inhibitor (SB203580) (B), and glycolysis rates were calculated by enolase assay as determined by release of the radioactive hydrogen on C-5 as water, measured via scintillation counting.

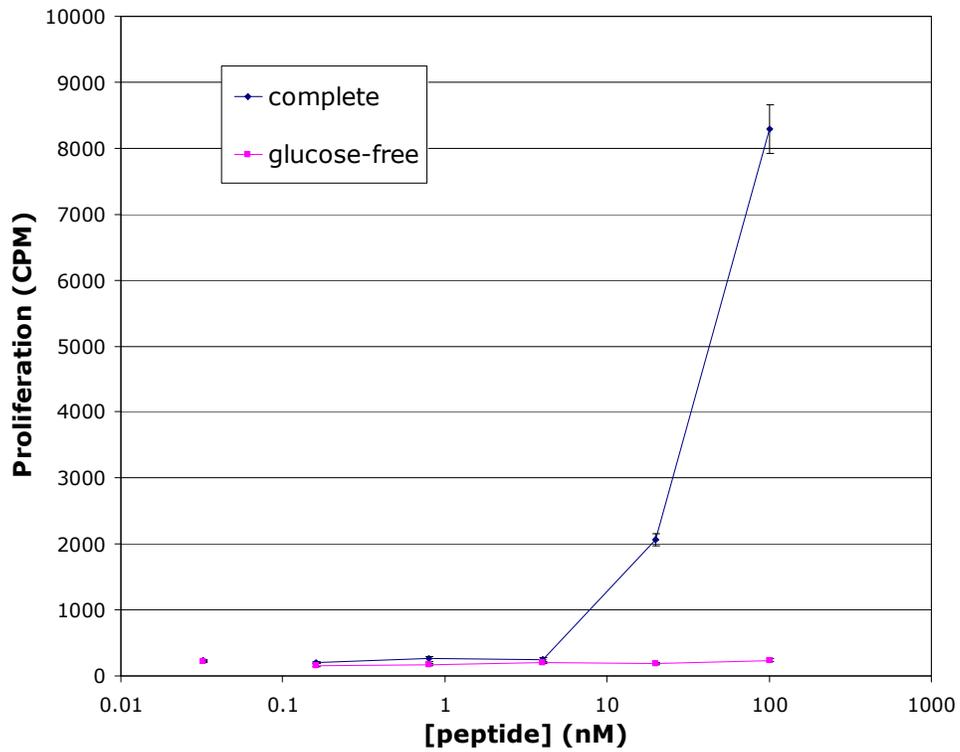


Figure 13. T Cell proliferation requires glucose. Unfractionated, RBC-depleted splenocytes from 2C TCR transgenic mice were stimulated for three days with titrated peptide in media containing dialyzed FBS and proliferation was assayed using tritiated thymidine. Data provided by Dr. Kenneth Frauwirth.

Although GLUT1 activity is important to transport glucose into cells, because the glucose remains uncharged it can easily interact with GLUT1 and be transported back out. The most important enzyme in increasing the presence of usable glucose in the cell is hexokinase. It phosphorylates glucose, which does two things. First, it traps it in the cell, as GLUT1 cannot transport out a negatively charged molecule. Second, adding the phosphate group turns glucose into a substrate for downstream enzymatic reactions such as glycolysis, pentose phosphate pathway and Krebs Cycle.

Hexokinase activity data for nonstimulated versus stimulated T cells are preliminary and are presented to showcase a trend in glucose metabolism, while Krebs Cycle and pentose phosphate pathway activity data for nonstimulated versus stimulated T cells have been confirmed. Fig. 14 represents one hexokinase assay in triplicate and was not repeated due to time constraints. Hexokinase activity was measured by fluorimetric detection of NADP to NADPH conversion in stimulated and nonstimulated T cell lysates in triplicate. Stimulated T cells showed upregulated hexokinase activity during the logarithmic phase of the assay which lasted about 2 minutes [Fig. 14].

Krebs Cycle is primarily catabolic, providing ATP directly, and also NADH and FADH₂ which are used by the mitochondria to make ATP. The pentose phosphate pathway is primarily anabolic, providing ribose-5-phosphate used to make DNA, erythrose-4-phosphate used in production of aromatic amino acids, and NADPH which is used in biosynthesis reactions. NADPH may also provide protection against

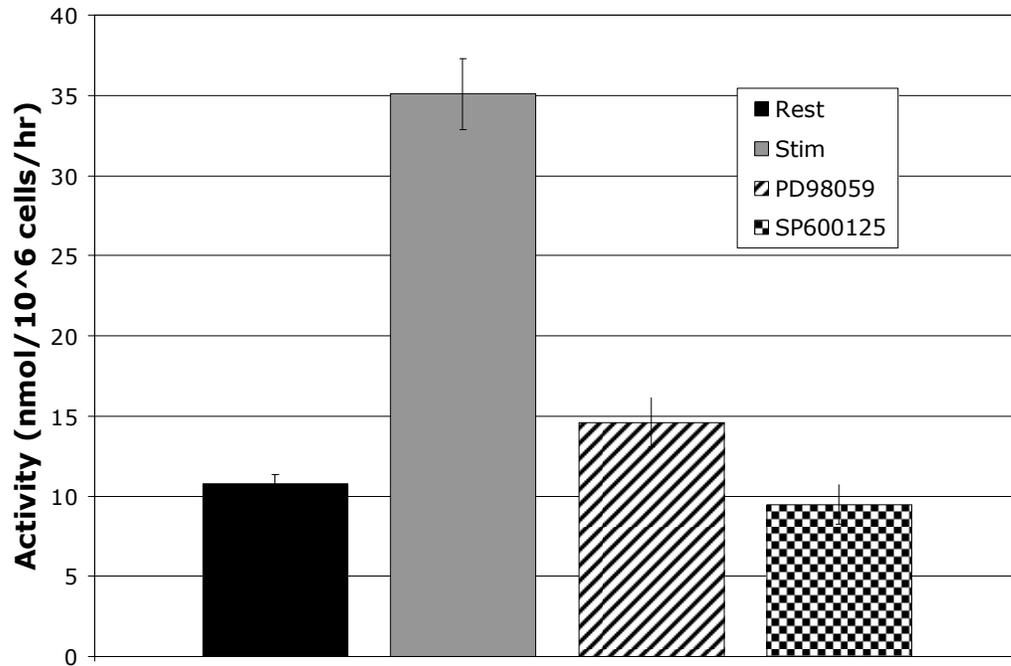


Figure 14. Inhibition of ERK, JNK, and p38 pathways lowers hexokinase activity.

Splenic T cells were stimulated for 24 hours in full-glucose RPMI with IgG or anti-CD3/anti-CD28 coated beads, +/- inhibitors, then collected and assayed for hexokinase activity. Enzyme activity was measured via a coupled fluorimetric reaction. Data received from Rebecca Miller and Dr. Kenneth Frauwirth.

free radicals produced by mitochondria whose activity is also upregulated during activation. The protective property of NADPH comes from its ability to return glutathione, an intracellular antioxidant, to its reduced state.

Krebs Cycle and pentose phosphate pathway were measured using a paired assay; two reactions were run separately, then analyzed for evolution of radioactive carbon dioxide. One reaction contained D-[1-¹⁴C]-glucose which had a radioactive carbon in the C1 position of glucose. This carbon is released as CO₂ during both Krebs Cycle and Pentose Phosphate Cycle. The second reaction contained only D-[6-¹⁴C]-glucose which had a radioactive carbon in the C6 position. This carbon is released as CO₂ only during Krebs Cycle. Krebs Cycle activity was determined directly from the count in the vial containing D-[6-¹⁴C]-glucose, and pentose phosphate pathway activity was determined by calculating how much of the radioactivity from the D-[1-¹⁴C]-glucose reaction was produced during the Krebs Cycle, then subtracting that amount from the total radioactivity produced. Stimulated T cells showed upregulated pentose phosphate pathway, but Krebs Cycle activity was not raised very much [Fig. 15].

Because carbon flux through Krebs Cycle results in ATP production and active cells need more ATP, it was expected that glucose use in Krebs Cycle would increase, but there was little change. This indicates that glucose is shunted away from Krebs Cycle during T cell activation, though the use of amino acids or other metabolites may be upregulated in this pathway. The pentose phosphate pathway was highly upregulated.

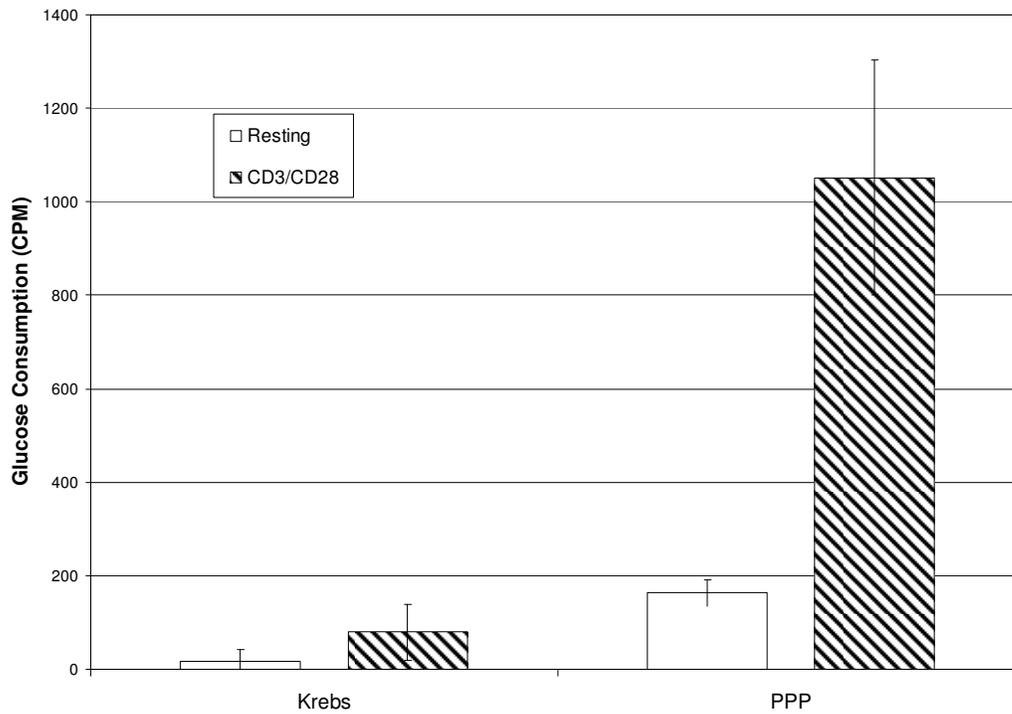


Figure 15. CD3/CD28 stimulation induces Krebs Cycle and Pentose Phosphate Pathway activity. Splenic T cells were stimulated for 24 hours with IgG or anti-CD3/anti-CD28 coated beads, and Krebs Cycle and Pentose Phosphate Pathway rates were calculated by CO₂-release assay as determined by release of the radioactive C-1 or C-6 as carbon dioxide, measured via scintillation counting. Activated T cells upregulate PPP relative to TCA.

Experiments with EL4 cells, a murine T cell lymphoma line, showed that they have an extremely high and constitutively upregulated flux of glucose through the pentose phosphate pathway, and very little flux through Krebs Cycle [Fig. 16]. The finding that T cells specifically upregulate only some of the pathways that metabolize glucose and the finding that cancer cells shunt glucose at a quick pace through the pentose phosphate pathway are strong evidence that the T cell actively and tightly controls how glucose is used in order to achieve a specific collection of end metabolites for optimal activation.

Preliminary Results Indicate That Glucose Flux May Respond Strongly to Signaling through the MAP Kinase ERK and JNK Pathways and to a Lesser Extent through the p38 Pathway.

Data for glucose flux through hexokinase, Krebs Cycle and pentose phosphate pathway measured under MAP kinase inhibition are preliminary and are presented to showcase a trend in glucose metabolism.

Inhibition of MAP kinases lowered hexokinase activity [Fig. 14]. This assay, in triplicate, was performed one time. The ERK kinase inhibitor U0126 and the JNK inhibitor SP600125 lowered uptake almost to resting levels, while the p38 inhibitor SB203580 lowered uptake to a lesser extent. Similar results were seen when the pentose phosphate pathway was measured [Fig. 16]. Fig. 16 represents one assay in singlet. Several other assays in triplicate and in singlet indicate that the data presented

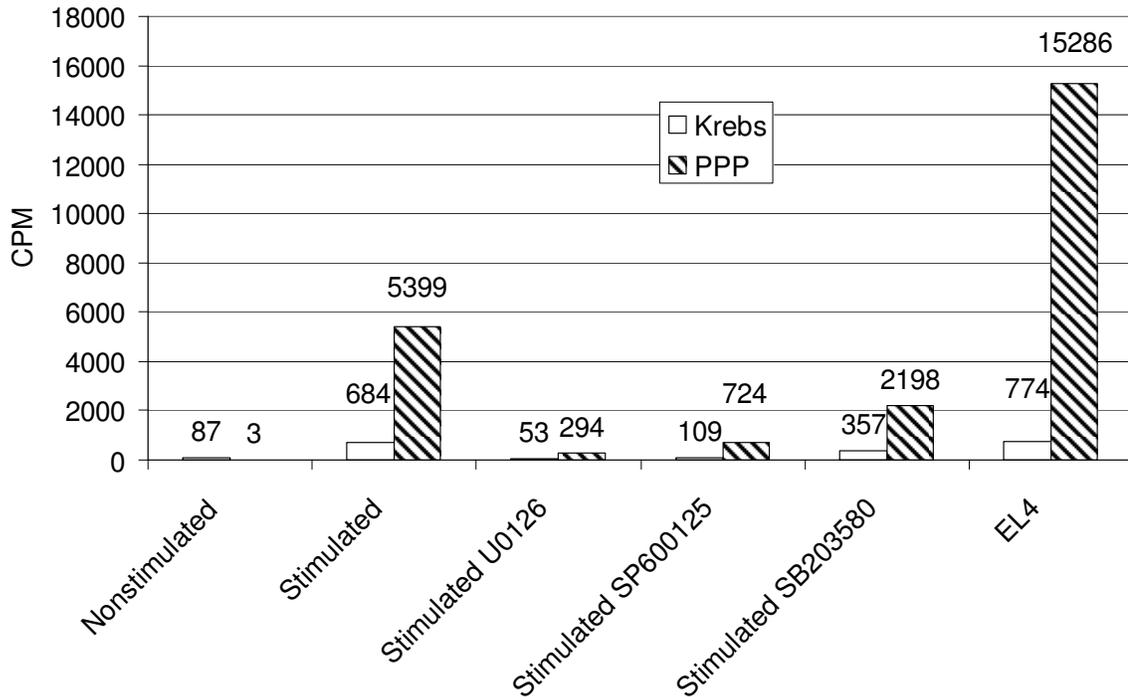


Figure 16. Inhibition of ERK, JNK and p38 pathways lowers Pentose Phosphate Pathway activity. Splenic T cells were stimulated for 24 hours with IgG or anti-CD3/anti-CD28 coated beads, + 2.6 μ M ERK inhibitor (U0126), or 50 μ M JNK inhibitor (SP600125), or 20 μ M p38 inhibitor (SB203580). Krebs Cycle and Pentose Phosphate Pathway rates were calculated by CO₂-release assay as determined by release of the radioactive C-1 or C-6 as carbon dioxide, measured via scintillation counting.

here are very representative of what we expect to see when this assay is confirmed. Krebs Cycle activity was slightly affected by MAP kinase inhibition, but because glucose use in Krebs Cycle was only minimally raised by TCR stimulation, the effects of the MAP kinases were not significant.

DISCUSSION

It is known that glucose metabolism increases upon initiation of activation in order to meet the increased metabolic needs of a proliferating, metabolically active cell. Little is known about the cellular control of metabolic changes that enable the cell to sustain its increased growth and proliferation programs. Our hypothesis was focused to determine if glucose metabolism was regulated by signal transduction initiated during T cell activation; we tested this hypothesis by administering and modulating TCR stimulation. We measured production of various metabolites of glucose and enzyme activity to determine which metabolic pathways were influenced by T cell stimulation. We then performed the same measurements while blocking specific signaling pathways. Our results show that ERK and JNK pathways appear to be determining factors because their inhibition downregulated glucose uptake and glycolysis. Inhibition of another MAPK pathway (p38) causes some downregulation of these activities but the effect is not as strong as with the ERK and JNK inhibitors. From this we can infer that the p38 pathway is less important in regulating glucose metabolism.

Emerging evidence is showing that not only do T cells need two signals in order to become activated, but that they also need an upregulated and sustained metabolism in order to successfully complete the activation process. Since glucose is a prime energy source, its use was a logical place on which to focus metabolism studies, and signaling pathways important to glucose metabolic regulation needed to be identified.

We limited our scope to MAP Kinases because these molecules are directly involved in T cell signaling. The three MAP Kinase families we studied were extracellular signal-regulated kinases ERK1 and ERK2; c-Jun amino-terminal kinases that are activated by stress JNK1, JNK2 and JNK3; and p38 isoforms alpha, beta, gamma and delta. (Chen et al. 2001) (Kyriakis and Avruch 2001).

The work presented here supports the hypothesis that metabolism is regulated directly via receptor-mediated signals. This is a change from previous views that catabolic derepression (i.e. depletion of nutrients through metabolism) was the signal that changed glucose use within the cell. We first showed that upregulated glucose uptake and glycolysis were two responses to T Cell Receptor signaling plus CD28 co-stimulation. Next we blocked several downstream MAP kinase signaling pathways and observed differing effects on glucose metabolism. ERK and JNK blockades were seen to control metabolism almost identically, while p38 blockade appeared to affect metabolism about half as strongly. These results taken together show that glucose metabolism can be regulated with T Cell Receptor signaling plus CD28 co-stimulation and also that blockade of this stimulation pathway results in the cell actively preventing changes in its glucose metabolism. It is interesting to note that blocking p38 stimulation did not have as great an effect as blocking the other two activation pathways. We believe this to be an important observation because other studies have shown that ERK and JNK behave similarly while p38 activity differs even though still clearly acting within the classical MAP kinase cascade. Comparing the DNA sequences of these three proteins could identify new motifs and enable

greater understanding of the human kinome. Also, that T Cell Receptor stimulation causes several pathways to be activated is important to note because this means that the cell could control each pathway separately, thereby allowing it to fine tune the repertoire of proteins produced.

The relationships between activating signals, MAP Kinases, and the final phenotype of a cell (whether activated or reacting to growth factors or other extracellular stimuli) are not easy to predict because activation pathways are more like webs and scaffolds than lines. MAPKs can be activated by many different stimuli, but in general, ERK1 and ERK2 are activated by growth factors and phorbol esters, while the JNK and p38 kinases are more responsive to stress stimuli like osmotic shock, radiation damage, or cytokine stimulation (Pearson et al. 2001). All MAP Kinase pathways consist of a cascade of activating kinases, but substrate selectivity may be modulated by specific interaction motifs on the protein that is being activated. Furthermore, MAPK cascade specificity is also mediated through interaction with scaffolding proteins which organize pathways in specific modules through simultaneous binding of several components (Pearson et al. 2001). These variables may be the cause of p38's weaker effect on changes in glucose metabolism. Many different studies ranging from detection of phosphorylated molecules to protein associations to kinase activity measurements could be done to characterize how the molecules we chose to study affected metabolism.

Knowledge of MAP Kinase effects on glucose metabolism could have many practical applications, not the least of which include chemotherapy and immunosuppression. Cancers and T cells that attempt to become activated have in common that their metabolisms are upregulated. Rapamycin has been shown to have anti-cancer actions (Guba et al. 2002), possibly because it affects glucose use within the cancer cells. Because cancer uses so much glucose, the environment inside a cancer mass may not be conducive to T cell activation. Knowledge of the importance of glucose to T cell activation will increase our understanding in this area. In addition, knowledge of T cell metabolism will reveal new uses for known drugs. For example, if the Pentose Phosphate Pathway is highly upregulated by cancer cells, but is not upregulated in activated T cells, this indicates that drugs that inhibit this pathway may safely be used to treat the cancer. In the same way HAART anti-retroviral therapy uses several chemicals to inhibit different stages of replication in HIV-infected cells, specific drugs that affect glucose metabolism could be combined with other drugs in order to shut down, modulate, or reroute nutrient metabolism in cancer cells.

In order to gain insight into the conclusions of this study, there are several important questions one could answer. Changes in glucose metabolism in human T cells are detectable within 6 hours of stimulation (Frauwirth et al. 2002), before cell cycle entry or growth begin. It has been suggested that T cells "survey" the environment (by detecting levels of metabolites, ATP, or nutrients) before they begin to proliferate in order to determine if there are sufficient raw materials to sustain activation. To

determine the cause-and-effect relationship between activation signals and metabolic signals it is necessary to dissect the pathways from start to finish.

To determine initial responses to stimulation in the absence of glucose, T cells could be stimulated in the presence or absence of glucose, and activation state of MAP Kinases assessed. One could also check proliferation, IL-2 production, and various surface activation markers such as CD25, and CD44 to see if other hallmarks of T cell activation are affected by glucose deprivation during a stimulation signal. Experiments to this effect have been performed by Dr. Kenneth Frauwirth, but not published.

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