Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Julia Veronica Wojdylo Date

Metabolism of CD4+ Th1 and Th2 Cells

By

Julia Veronica Wojdylo
Master of Science

Biology

_____

Victor G. Corces
Advisor

_____

David G. Lynn
Committee Member

_____

Periasamy Selvaraj
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

Metabolism of CD4+ Th1 and Th2 Cells

By

Julia Veronica Wojdylo

Advisor: Victor G. Corces, Ph.D.

An abstract of
A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science
in Biology
2014

Abstract

Metabolism of CD4+ Th1 and Th2 Cells
By Julia Veronica Wojdylo

The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that integrates diverse environmental inputs and regulates cell survival, proliferation, protein synthesis, and metabolism. mTOR, a member of the phosphoinositide 3-kinase related kinase family, associates with a variety of adapter proteins to form two distinct signaling complexes, mTORC1 and mTORC2, with disparate upstream regulators and downstream effectors.

T-lymphocytes are a critical component in cell-mediated immunity. T-cell receptor recognition of antigen alone fails to activate T-lymphocytes; a second antigen-presenting cell-derived costimulatory signal is necessary to prevent anergy and cell death. The immune microenvironment helps to guide $CD4^+$ T-lymphocytes to an effector lineage commitment, which is associated with dynamic changes in the function and metabolic program of the cell.

As a central signal integrator and regulator of many cellular metabolic programs, mTOR signaling is intimately associated with T-lymphocyte metabolism in the development, homeostasis, activation, and differentiation of T-lymphocytes. In the absence of mTOR, $CD4^+$ T cells fail to differentiate into effector cells and become $Foxp3^+$ regulatory T-cells. In addition, mTORC1 deficient cells fail to differentiate into Th1 or Th17 cells and mTORC2 deficient cells fail to differentiate into Th2 cells under appropriate skewing conditions.

Previous studies have shown that mTORC1 deficient cells are not highly glycolytic, yet the highly glycolytic Th2 cells can differentiate from mTORC1 deficient cells. To determine bioenergetic and biosynthetic differences between Th1 and Th2 cells, CD4+ T cells were isolated from 5C.C7 mice, stimulated for 48 hours under Th1 and Th2 skewing conditions, and then expanded with IL-2 for 5 days. Following expansion, cells were rechallenged with plate-bound anti-CD3 and anti-CD28 for 12 hours. Metabolic profiles of the cells were examined during the induction, resting, and rechallenge phase of Th1 and Th2 cells using RT-PCR and XF96 Analyzer. During induction and upon restimulation of Th1 and Th2 cells, it was found that both Th1 and Th2 cells upregulate glycolytic and pentose phosphate pathway machinery, and downregulate machinery involved in fatty acid oxidation. However, differences in levels of metabolic gene expression observed in Th1 and Th2 cells upon rechallenge suggest a more memory-like role for Th2 cells.

Metabolism of CD4+ Th1 and Th2 cells

By

Julia Veronica Wojdylo

Advisor: Victor G. Corces, Ph.D.

A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science
in Biology
2014

TABLE OF CONTENTS

LIST OF FIGURES

Page

Figure

LITERATURE REVIEW

Identification of mTOR

The mammalian target of rapamycin, mTOR, is an evolutionarily conserved serine/threonine kinase that integrates diverse environmental inputs and regulates broad aspects of cellular function, including growth, survival, metabolism, proliferation, and differentiation. It was first identified as the mammalian ortholog of two yeast genes, target of rapamycin 1 (TOR1) and target of rapamycin 2 (TOR2), inhibited by rapamycin, an antifungal drug extracted from the bacterium *Streptomyces hygroscopicus* (Weichhart, 2012). In contrast to yeast, mammalian genomes only encode a single TOR protein, mTOR, with an amino acid sequence identity of approximately 42% with the yeast TOR1 and TOR2 proteins (Hay & Sonenberg, 2004).

mTOR Structural Domains

mTOR, a member of the phosphoinositide 3-kinase related kinase (PIKK) family, is a 289 k-Da atypical serine/threonine kinase comprised of several conserved structural domains (**Figure 1**) (Powell et al., 2012). The N-terminus of mTOR is comprised of 20 tandem HEAT (huntingtin, elongation factor 3, subunit of PP2A, and TOR1) domains. The HEAT domain consist of repeats of two anti-parallel α-helices and two turns of approximately 47 amino acids linked together through flexible inter-unit loops that appear to function as scaffolding for the assembly of other molecular components (Andrade & Bork, 1995). Downstream of the HEAT repeats is the FAT (FRAP, ATM, and TRRAP) domain, the FRB (FKB12-rapamycin binding) domain, and the kinase

domain. The FRB domain is the binding site of the mTOR inhibitor, rapamycin. Rapamycin's mechanism of inhibition is not direct, but rather through the formation of a complex with its intracellular receptor, FK506-binding protein (FKBP12), which then binds the FRB domain on the C-terminus of TOR proteins (Chen et al., 1995). The kinase domain, similar in sequence to the catalytic domain of phosphatidylinositol 3-kinase (PI3K), confers the serine/threonine kinase activity of the protein and is the binding site for mTOR kinase-specific inhibitors (Hay & Sonenberg, 2004; Powell et al., 2012). The C-terminus contains a FAT domain (FATC domain) that appears to have a crucial role in the structural and cellular stability of mTOR. Deletion of amino acid residues in the FATC domain has been shown to almost completely abrogate the kinase activity of mTOR in vitro (Takahashi et al, 2000). In addition, Dames et al (2005) suggests that the redox state of the disulfide bond within the FATC domain may influence the degradation of mTOR.

Figure 1. Structural domains of mTOR (Powell et al., 2012)

mTOR Signaling Complexes

mTOR associates with two different sets of adapter proteins to form two functionally distinct signaling complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), with disparate upstream regulators and downstream effectors.

mTORC1 is composed of the regulatory-associated protein of mTOR (RAPTOR) and mammalian lethal with Sec13 protein 8 (mLST8); both adaptor proteins contain WD40 repeats, mediating protein-protein interactions. RAPTOR, comprised of a highly conserved N-terminal region, three HEAT repeats, and seven WD40 repeats, binds to the HEAT domains of mTOR and is indispensable for the function of mTORC1; the fundamental role of RAPTOR is evident in the drastic physiological consequences of RAPTOR knockouts, which are early embryonic lethal (Yang & Guan, 2007). In contrast to RAPTOR, mLST8 consists almost entirely of seven WD40 repeats and is thought to constitutively bind to the kinase domain of mTOR (**Figure 2**) (Yang & Guan, 2007).

Figure 2. Structures of mTORC1 adaptor proteins, RAPTOR and mLST8 (Yang &Guan, 2007)

mTORC1 may also associate with proline-rich Akt substrate 40kDa (PRAS40) and DEP domain-containing mTOR-interacting protein (DEPTOR), which inhibit mTORC1 activity (**Figure 3**) (Powell et al., 2012).

Figure 3. mTOR Complex 1 (mTORC1) (Powell et al., 2012)

Structurally, mTORC2 shares the adaptor proteins DEPTOR and mLST8 with mTORC1; however, this complex is distinguished from mTORC1 through the association of the scaffolding protein RAPTOR-independent companion of TOR (RICTOR), the protein observed with RICTOR (PROTOR), and mSIN1 proteins (**Figure 4**) (Powell et al., 2012).

Figure 4. mTOR Complex 2 (mTORC2) (Powell et al., 2012)

Another important distinguishing feature of the two signaling complexes is the pharmacological effects of rapamycin. Rapamycin complexes with FKBP12 and inhibits the activity of mTORC1 by binding to the FRB domain and blocking the interaction of mTOR with RAPTOR (Abraham & Wiederrecht, 1996; Powell et al., 2012). In contrast,

mTORC2 is regarded as rapamycin-insensitive. Although rapamycin-FKBP12 complex cannot bind to the FRB domain on the preformed mTORC2 (Jacinto et al., 2004), it can bind to newly synthesized mTOR and prevent the binding of RICTOR. Sarbassov et al (2006), showed that in specific tissues and cell types, prolonged exposure to rapamycin may inhibit mTORC2 activity by interfering with the assembly of mTORC2.

mTORC1 Signaling Pathway

mTOR complex 1 is a critical signal integrator of various environmental cues and master regulator of many metabolic programs through the induction of metabolic gene expression involved in glycolysis, *de novo* lipogenesis, and the oxidative branch of the pentose phosphate pathway (Düvel et al., 2010). It is also involved in mitochondrial biosynthesis and the regulation of autophagy (Powell et al., 2012). Through the coordination of environmental cues such as the presence or absence of growth factors, immunomodulatory factors, amino acids, energy levels, oxygen availability, and stress, to catabolic and anabolic processes in the cell, mTORC1 provides a means of maintaining cellular homeostasis and regulating cellular function.

An essential component of mTORC1 activation is the Ras homolog enriched in brain (RHEB) protein, a small RAS-like guanosine triphosphatase (GTPase) anchored to the surface of the lysosome that can bind directly to mTOR (Sancak et al., 2008). The GTP-bound form of RHEB is able to activate the kinase activity of mTOR (Long et al., 2005). RHEB is tightly regulated by the hetero-dimeric Tuberous Sclerosis Complex (TSC), composed of TSC1 and TSC2 subunits, a GTPase-activating protein (GAP). Therefore, TSC increases RHEB's intrinsic rate of GTP hydrolysis thus inactivating

RHEB and mTORC1. Integrating upstream signals, TSC functions as a molecular switch to regulate mTORC1 kinase activity through the activity RHEB.

Many growth factors, cytokines, and other co-stimulatory factors activate phosphatidylinositol-3-kinase (PI3K) via interaction with cell surface receptors. PI3K catalyzes the formation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3). The proteins Akt, also known as serine/threonine protein kinase B (PKB), and 3-phophoinositide-dependent protein kinase-1 (PDK1) contain a pleckstrin homology (PH) domains, enabling these proteins to bind to PIP3 (Toker & Newton, 2000). PIP3 is restricted to the cell membrane and therefore recruits and localizes Akt and PDK1 to this region of the cell, allowing for PDK1 to phosphorylate Akt at the activation loop site T308 (threonine 308) and S473 (serine 473) (Yang & Guan, 2007). Dual phosphorylation of Akt allows for its full activation. Once activated, Akt phosphorylates TSC2 on multiple sites, inhibiting its GAP activity and leading to subsequent activation of mTORC1 kinase activity (Inoki et al., 2002).

Other signaling pathways can also regulate the activity of TSC. In the AMPK-mediated pathway, the ratio of ATP:AMP, reflecting the intracellular energy stores, modulates AMP-activated kinase (AMPK) activity. Decreased ATP:AMP levels activates AMPK, which can then phosphorylate TSC2 on S1345. This phosphorylation by AMPK may serve as a priming phosphorylation for glycogen synthase kinase 3β (GSK3β) to catalyze subsequent phosphorylations on S1341 and S1337 (Inoki et al., 2002). Unlike Akt, these phosphorylations enhance the ability of TSC to inhibit mTORC1 activity. Wnt signaling inhibits GSK3β, promoting mTORC1 activity (Powell et al, 2012). Another important pathway is the MAPK pathway. The activation of the

RAS-mitogen-activated protein kinase (MAPK) pathway leads to phosphorylation of TSC via ERK1/2. This phosphorylation inhibits TSC and also promotes mTORC1 activity.

TSC may also be activated in hypoxic conditions by hypoxia-induced factor protein regulated in the development of DNA damage response 1 (REDD1) (Powell et al, 2012). It is evident that TSC plays a major role in modulating mTORC1 activity, and this is further exemplified by studies showing that a loss of TSC1 may lead to enhanced basal mTORC1 activity (Yang et al., 2011).

mTORC1 regulation also occurs via TSC-independent pathways. In addition to AMPK-mediated phosphorylation of TSC2, Gwinn et al (2008) show that AMPK can also directly phosphorylated RAPTOR on two highly conserved serine residues, S722 and S792, and causing inhibition of mTORC1 during energy stress. Similarly, Akt is also capable of modulating mTORC1 activity through substrates other than TSC2. Akt can phosphorylate PRAS40 near its C-terminus on T246 and relieve its inhibitory effect on mTORC1 (Kovacina et al., 2003).

Furthermore, mTORC1 activation requires the presence of amino acids (Powell et al., 2012). As previously mentioned, RHEB is localized at the surface of the lysosome. In order for mTORC1 to be activated by RHEB, mTORC1 needs to be translocated to the lysosomes. This translocation is controlled by hetero-dimeric Rag GTPases, composed of RagA or B in complex with RagC or D, and the Ragulator, a pentameric complex that functions as a guanine nucleotide exchange factor (GEF) and lysosomal anchor for the Rag GTPases (Bar-peled et al., 2012). The interaction between the RAG GTPases and the

Ragulator is dependent upon the presence of amino acids (Kim et al., 2008). According to Powell et al (2012) the most potent activators of mTORC1 are the branch chain amino acids (BCAA) such as leucine (**Figure 5**).

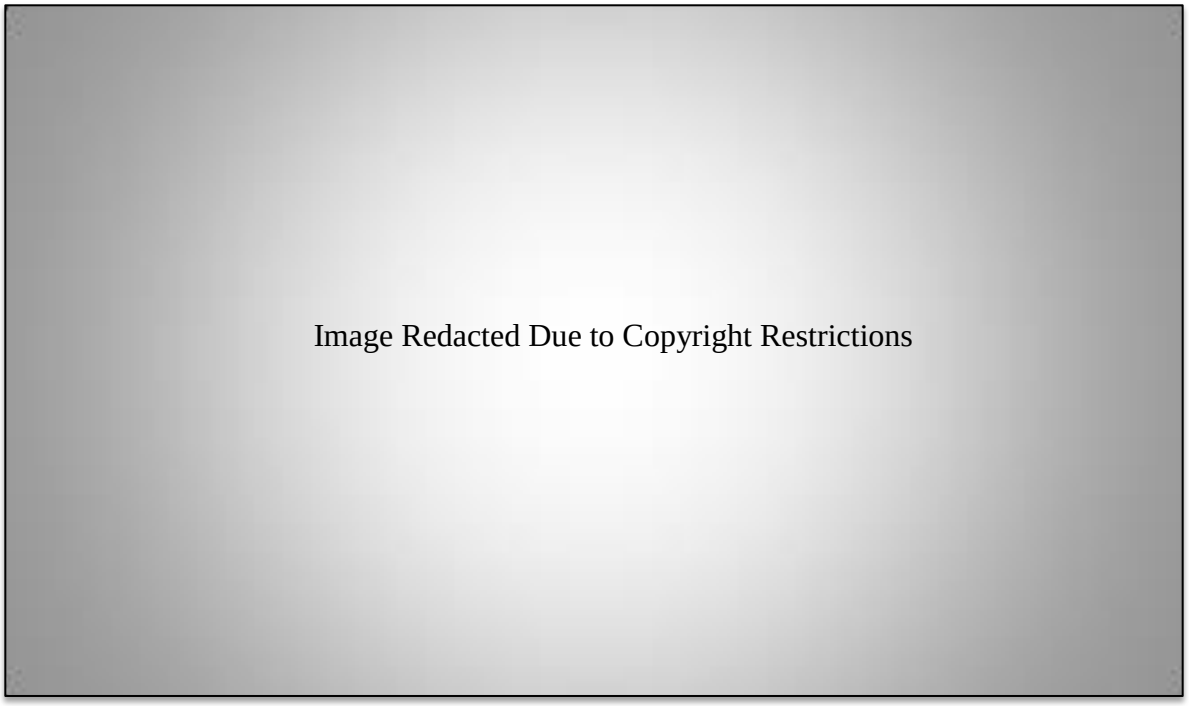

Figure 5. Upstream signaling of mTORC1 (Powell et al., 2012)

Canonical substrates of mTORC1 are the ribosomal S6 kinase 1, S6K1, and the eukaryotic initiation factor 4E, 4E-BPI. Both S6K1 and 4E-BPI are involved in the regulation of cap-dependent translation initiation. In order to initiate translation, the eukaryotic translation initiation factor 4F complex (eIF4F) must first assemble on the 5' cap structure of mRNA in order to recruit the 40S ribosomal subunit to mRNA. The formation of the eIF4F complex is dependent upon three initiation factors, eIF-4E, eIF-4G, and eIF-4A. 4E-BPI inhibits eIF-4E from binding to eIF-FG and thus prevents the assembly of the complex. mTORC1-mediated phosphorylation of 4E-BPI causes this protein to dissociate from eIF-4E, allowing for the initiation of translation, and

mTORC1-mediated phosphorylation of S6K1 recruits eIF-4B to the eIF-4F complex and enhances the RNA helicase activity of eIF-4A (Ma & Blenis, 2009).

Key transcription factors upregulated through mTORC1 activation include HIF-1α, Myc, SREBP1, PPARα, and PPARγ. HIF-1, hypoxia inducible factor, is a hetero-dimeric protein composed of an inducible α subunit and a constitutively expressed β subunit. HIF-1 controls the glycolytic pathway by increasing expression of gene targets such as the glucose transporter 1, GLUT-1 and lactate dehydrogenase A (LDH-A) (Chen et al., 2001). HIF-1 also induces the expression of pyruvate dehydrogenase kinase 1 (PDK-1), reversing the activity of pyruvate dehydrogenase, an enzyme that converts pyruvate to acetyl-CoA and carbon dioxide, thus enforcing the glycolytic pathway (Waickman & Powell, 2012).

Myc, a very strong proto-oncogene, controls metabolic pathways fundamental to cell growth and proliferation, including glycolysis, glutaminolysis, and fatty acid oxidation (Waickman & Powell, 2012). Upon TCR stimulation, Myc expression is rapidly induced in order to facilitate the metabolic demands of T cell proliferation. Myc is also a transcriptional repressor of TSC2 and thus is involved in a feed-forward loop that increases mTORC1 activity and further enhances Myc expression (Waickman & Powell, 2012).

Peroxisomal proliferator-activated receptor α, PPARα, is a ligand-activated transcription factor that senses endogenous fatty acids to regulate mitochondrial fatty acid β-oxidation, fatty acid uptake, and glucose homeostasis (Forman et al., 1997). The translation of PPARα and its transcriptional activity is decreased by mTORC1 activity.

PPARγ, a homolog of PPARα, is also capable of regulating lipid metabolism and glucose homeostasis; however, in contrast to PPARα, PPARγ transcription is enhanced by mTORC1 activity (Waickman & Powell, 2012).

SREBP1, sterol regulatory element-binding protein 1, is a member of a family of basic helix-loop-helix transcription factors that regulate many genes implicated in lipid biosynthesis and the pentose phosphate pathway (Powell et al., 2012). Furthermore, mTORC1 activity increases the transcriptional activity of the PPARγ coactivator, PGC1a, which has been shown to control mitochondrial gene expression (such genes involved in the tricarboxylic acid cycle and oxidative phosphorylation) through transcriptional factors that include oestrogen-related receptor a (ERR-a), and nuclear respiratory factors (NRFs) (Cunningham et al., 2007) (**Figure 6**).

Figure 6. Key transcriptional factors downstream of mTORC1 (Waickman & Powell, 2012).

mTORC2 Signaling Pathway

mTOR complex 2 is also involved in numerous cellular functions including cell survival, metabolism, proliferation, and cytoskeleton organization (Powell et al., 2012). However, its upstream regulators are much less well-defined in contrast to mTORC1. The canonical substrates of mTORC2 are Akt, serum and glucocorticoid-inducible kinase 1 (SGK1), and protein kinase C α (PKCα) (García-martínez & Alessi, 2008; Guertin et al., 2006).

mTORC2-mediated phosphorylation of Akt is distinct from PDK1-dependent phosphorylation of Akt at the T308 residue upstream of mTORC1 signaling. The mTORC2 signaling pathway phosphorylates Akt at S473 (Bayascas & Alessi, 2005). Activation of Akt through mTORC2 kinase activity leads to inactivation of several transcription factors, such as Forkhead box protein O1 (FOXO1) and FOXO3, through their phosphorylation and subsequent sequestration to the cytoplasm (Powell et al., 2012).

SGK1, a member of the AGC (protein kinase A/protein kinase G/protein kinase C) family is activated by the phosphorylation of two residues: a threonine in the T-loop of the kinase domain and a serine within the C-terminal hydrophobic motif (S422) (Garcia-Martinez & Alessi, 2008). Mechanistically, SGK1 is similar to Akt in that it is also capable of phosphorylating and sequestering FOXO family members to the cytoplasm (Brunet et al., 2001).

PKC is a serine/threonine kinase that is regulated by mTORC2. mTORC2 has been shown to phosphorylate PKC at the turn motif (TM) and the hydrophobic motif (HM), both of which are essential to the function of PKC (Ikenoue et al., 2008).

Phosphorylation of PKCα at these motifs leads to actin reorganization (Powell et al., 2012).

Regulation of T cell Differentiation, Activation, and Function by mTOR

The T cells are a subset of lymphocytes that are a critical component in cell-mediated immunity, a response important for the elimination of bacterial and viral infections as well as parasites and tumors. One of the most remarkable features of these lymphocytes is their vast diversity of antigen-specific receptors stochastically generated from a limited amount of genes during their development. T-cell receptor (TCR) recognition of antigen alone, however, fails to activate the T cell and can lead to a tolerogenic response. A second antigen presenting cell (APC) derived costimulatory signal is necessary in order to generate a robust immune response. Once activated, the naïve T cell can differentiate into many distinct subsets, each with a specific effector function. It is the immune microenvironment that helps guide the T cells to a specific effector lineage commitment and thus dictates the outcome of antigen recognition.

The commitment to a specific effector lineage is associated with dynamic changes in the metabolic program of the cell as distinct functions of cells are in part defined by distinct bioenergetic and biosynthetic requirements. As a central signal integrator and regulator of many cellular metabolic programs, mTOR signaling is intimately associated with T cell metabolism in the development, homeostasis, activation, and differentiation of T cells.

Naïve T cells

After surviving the selection process in the thymus during its developmental stage, naïve T cells enter the peripheral pool of lymphocytes and migrate through the secondary lymphoid organs seeking to encounter its cognate antigen. The metabolic demands for basic cell functions and migration are relatively low. Naïve cells employ a catabolic program, utilizing fatty acid β-oxidation, the tricarboxylic acid cycle, and autophagy for the efficient generation of adenosine triphosphate (ATP) and biosynthetic precursors (Waickman & Powell, 2012; Macintyre & Rathmell, 2013). Interestingly, naïve cells also express low levels of mTOR activity during this stage. Wu and colleagues (2011) demonstrated the importance of mTOR in maintaining the quiescence and survival of T cells in the absence of antigen presence; T cell specific deletion of TSC1 resulted in increased mTOR activity, and spontaneous activation of naïve T cells.

T cell Activation

The first 24hrs upon activation of the T cell are marked by a growth phase and a drastic change in metabolic profile, subsequently followed by massive clonal expansion and differentiation (Wang et al., 2011). Cells may undergo a round of replication every 4-6 hours and such an expansion requires both energy and *de novo* synthesis of many macromolecules (Van stipdonk et al., 2003). Under aerobic conditions, most differentiated cells will exploit the highly efficient TCA cycle in order to generate ATP; interestingly, activated lymphocytes utilize oxidative glycolysis instead in order to use the by-products of this pathway as substrates for protein, nucleotide, and lipid

biosynthesis (the Warburg effect). To meet these metabolic demands, T cells increase their uptake and consumption of glucose, glutamine, and amino acids shifting to a more glycolytic and anabolic profile, and downregulate gene expression involved in fatty acid oxidation. The shift in the metabolic profile of T cells following activation appears to be dependent upon the transcription factors HIF-1 and Myc, both regulated by mTOR. (Waickman & Powell, 2012). Failure to upregulate glycolytic machinery leads to a decreased rate of proliferation and drastically affects the effector lineage (Waickman & Powell, 2012).

It is important to note that studies suggest that the changes in the metabolic profile of the T cells are not merely a result of T cell activation, but rather function to promote T cell activation (Fox, Hammerman, & Thompson, 2005). Zheng and colleagues (2009) demonstrate that upon the engagement of the TCR in the context of costimulation, by blocking leucine, glucose, and energy metabolism T cell activation is diminished; also the inhibition of metabolic pathway promote T cell anergy in differentiated T cells.

As suggested earlier, mTOR plays a pivotal role in the regulation of T cell function, activation, and differentiation via its regulation of cellular metabolism. The ligation of the TCR results in a signaling cascade that includes the activation of PI3K, Akt, and subsequent induction of the activity of mTOR. Stimulation of costimulatory receptors such as CD28 and OX-40 further enhances activation, while inhibitory receptors such as CTLA-4 and PD-1 inhibit the PI3K and Akt, antagonizing mTOR activity (Frauwirth et al., 2005; Parry et al., 2005). Cytokines, chemokines, and other various immunologically relevant factors also have been shown to induce mTOR activity.

Signals in immune microenvironment may be integrated by mTOR into a response and thus affecting function to reflect the conditions of the environmental milieu.

The importance of mTOR in regulating immune response is exemplified by studies showing the inability of RHEB-/- T cells, mTORC1-deficient, cells to differentiate into Th1 or Th17 cells and RICTOR -/- T cells, mTORC2-deficient, to differentiate into Th2 cells. Furthermore, stimulated mTOR -/- T cells spontaneously induce Foxp3 and differentiate into regulatory t cells (Delgoffe et al., 2009). Each T cell subset has a unique cytokine secretion and transcription factor expression; these findings suggest that the T cell subsets also have distinct metabolic profiles, regulated in part by mTOR.

The Th1 CD4+ T cell lineage, characterized by the cytokine interferon gamma (IFN-γ) production and the transcription factor T-box expressed in T cells (Tbet) expression, is dependent upon interleukin (IL)-12-induced signaling via signal transducer and activator of transcription 4, STAT4 (Szabo et al., 2000; Waickman & Powell, 2012) (**Figure 7**).

Figure 7. Differentiation of Th1, Th2, and Th17 cells (Zhu, et al., 2010)

STAT4 is a member of the family of STAT proteins, which are activated by Janus kinases through phosphorylation on specific tyrosine residues, allowing the STAT monomers to then dimerize via SH2 domains and translocate into the nucleus to activate transcription of their target genes. STAT4 can directly induce cytokine IFN-γ production and expression of the receptor IL-12Rβ2 and the transcription factor T-bet. STAT4 is positively regulated by IFN-γ and negatively regulated by IL-4 and GATA3 (Zhu et al., 2010). Th1 CD4+ T cells have a highly glycolytic profile and express high levels of the glucose transporter, GLUT-1 (Michalek et al., 2011). The dependence of these cells upon this metabolic pathway may be demonstrated through the effects of the inhibition of glycolysis by competitive analogs, such as 2-deoxy-D-glucose (2-DG), in which these cells fail to secrete IFN-γ (Zheng et al., 2009). In addition to the requirement of glucose, the development of the Th1 phenotype is dependent on the presence of the mTORC1 signaling complex as studies show that RHEB-/- T cells exhibit decreased STAT4 and STAT3 signaling in response to IL-12 and IL-6 and fail to differentiate into the Th1 CD4+ T cells. The RHEB -/- T cells had increased levels of suppressor of cytokine signaling (SOCS) 3, an inhibitor of STAT3 and STAT6, possibly attributing to the decrease in STAT phosphorylation (Delgoffe et al., 2009) (**Figure 8**).

Figure 8. Role of mTOR in CD4+ T cell differentiation (Powell et al., 2012)

The Th2 CD4+ T cell subset is also highly glycolytic and expresses a high level of the glucose transporter, GLUT-1. This subset of T cells is characterized by the production of cytokines IL-4, IL-5, and IL-13, and the expression of the transcription factor, GATA3. Th2 differentiation is achieved through IL-4-mediated STAT6 phosphorylation, which induces GATA3 expression (Takeda et al., 1996). Strong IL-2-mediated STAT5 activation is also required for Th2 differentiation (Cote-sierra et al., 2004; Gilmour, Pine, & Reich, 1995). Unlike their Th1 counterparts, Th2 cells are not dependent upon the mTORC1 signaling complex for their development; however, Th2 cells fail to develop in the absence of the mTORC2 signaling complex as shown by studies using RICTOR -/- T cells, possibly attributed to a decrease in IL-4-induced STAT6 activation (Waickman & Powell, 2012; Delgoffe et al., 2011). Why $CD4^+$ T cells can differentiate into the highly glycolytic Th2 cells without the signaling capacity of mTORC1 is an area of active investigation.

The Th17 CD4+ T cell effector lineage has a crucial role in the clearing of certain bacterial and fungal pathogens and autoimmunity. This effector lineage may be generated in the presence of transforming growth factor (TGF)-β, IL-6, and IL-21 (Qin et al., 2009). IL-6 and IL-21 induce activation of STAT3, a transcription factor crucial for the development of the Th17 cells. Although IL-6 and IL-21 also induces the expression of SOCS3, a negative regulator of the Th17 development, Qin and colleagues (2009) demonstrate that TGF-β inhibits IL-6-induced SOCS3 promoter activity in T cells. The Th17 CD4+ T cell lineage is distinguished by secretion of the cytokines IL-17A, IL-17F, and IL-22 and the expression of the transcription factor RORγt. Also like the Th1 and Th2 CD4+ T cells, Th17 cells are highly glycolytic. Inhibition of the mTORC1 signaling complex by rapamycin and the genetic deletion of RHEB from T cells both result in a loss of Th17 differentiation. No effect on the development of the Th17 cells appears to be seen in mTORC2 signaling complex deficient T cells (Waickman & Powell., 2012).

According to Shi and colleagues (2011), HIF-1α expression is dramatically induced in Th17 following activation. This finding is not surprising since HIF-1α is involved in the expression of genes in the glycolytic pathway and the highly glycolytic Th17 cells appear to rely more on this metabolic pathway than other T cell subsets (Waickman & Powell, 2012). In addition, activation of SREBP1, implicated in lipid biosynthesis and the pentose phosphate pathway, seems to impair Th17 development (Cui et al., 2011; Waickman & Powell, 2012).

The regulatory T cells are essential for maintaining peripheral tolerance, preventing autoimmune diseases, and suppressing excessive immune responses

deleterious to the host. The master regulator for Tregs is the transcription factor foxhead box p3 (Foxp3). In contrast to the Th1, Th2, and Th17 CD4+ T-cell subsets, regulatory T cells (Tregs) utilize fatty acid β-oxidation and mitochondrial respiration in order to meet the metabolic demands. Pharmacological studies support this observation; inhibition of glycolysis following treatment with 2-DG or rapamycin results in the enhanced generation of Foxp3+ T cells and inhibition of fatty acid β-oxidation with etomoxir, an irreversible carnitine palmitoyltransferase I inhibitor, demonstrates the opposite effect (Waickman & Powell, 2012). Furthermore, PPARγ facilitates Treg generation, while HIF-1 antagonizes the development of Foxp3+ Treg cells. Selective deletion of either mTORC1 signaling complex or mTORC2 signaling complex does not result in the generation of Tregs; the inhibition of both signaling complexes is required for the generation of this T cell subset (Delgoffe et al., 2011).

EXPERIMENTAL PROCEDURES

Mice

Mice were kept in accordance with the guidelines set forth by the Johns Hopkins University Institutional Animal Care and Use Committee in pathogen-free conditions prior to experimental manipulations. C57BL/6 mice were purchased from Jackson Laboratories and 5C.C7 mice bred to a Rag2-/- background were purchased from Taconic Farms.

Isolation of **CD4+ T lymphocytes**

Lymphocytes were isolated from the axillary, brachial, and inguinal lymph nodes and the spleen. Cells were resuspended in 1mL Ack red blood cell lysis buffer (NH_4Cl), incubated at room temperature for 2 minutes, and washed with 10mL phosphate-buffered saline, PBS. Following the centrifugation (5 minutes at 300 x g), cells were counted in an appropriate volume of PBS and the cell solution was spun down again. The supernatant was aspirated and the cell pellet was resuspended in 300µL of magnetic sorting buffer (MACS, Miltenyi Biotec) and 50µL of Biotin-Antibody cocktail (MACS CD4+ T cell Isolation Kit, Miltenyi Biotec) per 10^8 cells. MACS buffer was prepared using PBS, pH 7.2, 0.5% bovine serum albumin (BSA), and 2mM EDTA by diluting MACS BSA Stock Solution (Miltenyi Biotec) 1:20 with autoMACS™ Rinsing Solution (Miltenyi Biotec). Cells were incubated at 4 degrees Celsius for 20 minutes. Afterwards, 300µL of MACS buffer and 100µL of Anti-Biotin Microbeads (MACS CD4+ T cell Isolation Kit, Miltenyi Biotec) were added per 10^8 cells and the solution was incubated at 4 degrees Celsius for 10 minutes. 10mL of MACS buffer were added following the

incubation period and the solution was centrifuged at 4 degrees Celsius for 5 min at 300 x g. LS MACS column was placed into the MACS™ Separator and equilibrated with 5mL of MACS buffer, discarding the flow. Cell pellet was resuspended in 1mL of MACS buffer and added to the column, collecting the flow through (negatively selected fraction). LS MACS column was washed 3x with 3mL of MACS buffer, adding buffer each time the column reservoir was empty and collecting all the flow through. Cell were spun down, counted, and resuspended in PBS or culture media, as necessary.

T cell Stimulation and Skewing

Unless specified otherwise, T cells from 5C.C7 mouse strain were stimulated with 5 μM of pigeon cytochrome c, PCC, peptide. T cells from all other mouse strains were stimulated using anti-CD3 (5 μg/ml) diluted in PBS (flat-bottomed plates were coated with this solution for 3 hours in 37 degree Celsius incubator), and anti-CD28 (2 μg/ml). Skewing conditions were as follows: Th1, IL-12 (10 ng/mL), IFN-γ (10 μg/mL) and anti-IL-4 (5 μg/mL), with IL-2 (1 ng/mL) during expansion; Th2, IL-4 (10 ng/mL), anti-IL-12 (5 μg/mL) and anti-IFN-γ (5 μg/mL), with IL-2 (1 ng/mL) during expansion; Th17, TGF-β (10 ng/mL), IL-6 (10 ng/mL), anti-IFN-γ (5 μg/mL), and anti-IL-4 (5 μg/mL), with IL-23 (1ng/mL) during expansion; Treg, TGF-β (10 ng/mL), IL-2 (1 ng/mL), anti-IFN-γ (5 μg/mL), anti-IL-4 (5 μg/mL), and anti-IL-12 (5 μg/mL), with IL-2 (1 ng/mL) during expansion.

Ficoll Extraction

Cells were collected, washed with 5 mL of PBS, centrifuged at 4 degrees Celsius for 5 min at 300 x g, and the supernatant was aspirated. The cell pellet was then suspended in media (same media cells were cultured in) and Ficoll-Paque Plus (GE Healthcare) was carefully added to the bottom of the conical tube in a 1:1 ratio. The cell suspension was then centrifuged at 4 degrees Celsius for 20 min at 600 x g. The interface between the two liquid layers of the cell suspension, which contains the live cells, was removed. Appropriate media was added and the cell solution was washed 2 x, resuspended in media, and the cells were counted.

Total RNA Extraction

Samples were transferred to Eppendorf® Safe-lock microcentrifuge tubes. To each sample, 800µL of TRIzol® Reagent (Life Technologies) was added and incubated at room temperature for 10 minutes. Afterwards, 160µLof chloroform was added, the sample tubes were vigorously shaken by hand for 15 seconds, and incubated again at room temperature for 10 minutes. Following the incubation period, the samples were centrifuged at 4 degrees Celsius at 12,000 x g for 20 min. The mixture separated into a lower pink, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The RNA, which remains exclusively in the aqueous phase, was carefully extracted and transferred into a fresh tube. The RNA was precipitated from the aqueous phase by adding 2µL of glycogen and 400µL of isopropanol, incubating at room temperature for 10 minutes, centrifuging the sample at 4 degrees Celsius for 10 min at 12,000 x g, and removing the supernatant (without disturbing the pellet). The RNA pellet was washed 1x

with 70% ethanol, mixed well, and centrifuged at 4 degrees Celsius at 7,500 x g for 5 minutes. The supernatant was removed and the RNA pellet was air dried (not letting the pellet over dry). RNA was dissolved in RNase-free water and incubated at room temperature for 10 minutes. The purity and integrity of the extracted RNA was measured using the ND-1000 NanoDrop Spectrophotometer (ThermoScientific).

cDNA Synthesis

cDNA was synthesized from extracted RNA in accordance with the manufacturer's protocol (New England BioLabs). For each sample, 800 mg of RNA (concentration determined using ND-1000 NanoDrop Spectrophotometer, ThermoScientific) and nuclease-free water was added to a PCR tube for a total volume of 9 µL. Random hexamers, dNTP mix, and nuclease-free water were added into an Eppendorf® in a 1:1:1 proportion, mixed well, and 6 µL of this mixture was then transferred to each PCR tube. The PCR tube was incubated at 65 degrees Celsius for 5 min and then at 4 degrees Celsius for 2 min in a thermocycler. 5x M-MuLV Reverse Transcriptase Buffer and M-MuLV Reverse Transcriptase (RNase H^-) were added into a new Eppendorf® in a 4:1 proportion, mixed well, and 5 µL of this mixture was then transferred to each PCR tube. The PCR tube was incubated at 25 degrees Celsius for 10 min, 42 degrees Celsius for 60 min, and 90 degrees Celsius for 10 min in a thermocycler. Finally, 2 µL of RNase H^+ to each PCR tube and incubated at 37 degrees Celsius for 20 min.

Real-Time PCR

Real-time PCR was performed in accordance with the manufacturer's protocol (Applied Biosystems). The real-time PCR assay was carried out in a 20 µL reaction mixture containing 10 µL of 2x TaqMan® Universal PCR Master Mix, 7 µL of RNase-free water, 1 µL of TaqMan® Gene Expression Assay (primary gene of interest), 1 µL of TaqMan® Gene Expression Assay (18s), and 1µL of sample cDNA in MicroAmp® Optical 96-Well Reaction Plate covered with MicroAmp® Optical Adhesive Film. The thermal cycling conditions were as follows: an initial denaturation step at 95 degrees Celsius for 10 min, 40 cycles of PCR amplification at 95 degrees Celsius for 15 seconds, and 60 degrees Celsius for 1 minute. Each sample was run in triplicate. Relative RNA abundance was determined based on control 18s RNA abundance.

Seahorse Assay

The wells of the XF96 Sensor Cartridge (calibrant plate) were filled with 200 µL of XF Calibrant (Seahorse Bioscience). Parafilm® was wrapped around the plate to ensure hydration and placed in a 37 degree Celsius non-CO_2 incubator for approximately 12 hours. The wells of the XF96 Cell Culture Microplate (sample plate) was coated with 50 µL of Poly-D Lysine to allow cells to adhere to the bottom of the plate. Cells were harvested, washed 2x with XF Media, and resuspended in XF Media, with or without additives, at a specific concentration. The concentrations of cells used were in the range of 50,000 cells per 162 µL of solution to 300,000 cells per 162 µL of solution; the exact concentration of cell solution used was dependent upon on the experiment and is indicated in the experimental results section along with the exact composition of the XF

Media (if additives were included). The sample plate coated earlier with Poly-D Lysine was then washed 3x with 100 µL of nuclease-free water and 162 µL of cell solution was added to each respective well. Wells not designated to receive cell were filled with 162 µL of XF Media. The sample plate was then gently centrifuged for 1 minute and placed in the non-CO_2 incubator at 37 degrees Celsius.

Drugs utilized for the metabolic assays were prepared in the following concentrations: Oligomycin, 1 µM; FCCP, 0.5 µM; AOAA, 1mM; UK5099, 50 µM, Etomoxir, 200 µM; Rapamycin, 1 µM; and PP242, 1 µM. The calibrant plate, containing four ports (A, B, C, and D) that can each inject into a well at times specified by the user of the XF96 Analyzer, was then removed from the non-CO_2 incubator and the designated ports of the designated wells were loaded with 18 µL of the drug (listed in the experimental results section). For metabolic assays in which cells were stimulated (or restimulated) during the assay, a solution consisting of 2 µg/mL of anti-CD3, 2 µg/mL of anti-CD28, and 1 µg/mL of IgG was prepared and loaded into designated ports of the experimental wells. Once a protocol was generated on the XF Analyzer, the calibrant plate was inserted into the instrument followed by the sample plate (immediately after the completion of the calibration).

EXPERIMENTAL RESULTS

To determine bioenergetic and biosynthetic differences between Th1 and Th2 cells, CD4+ T cells were isolated from 5C.C7 mice, stimulated for 48 hours under Th1 and Th2 skewing conditions, and then expanded with IL-2 for 5 days. Following expansion, cells were rechallenged with plate-bound anti-CD3 (1µg/mL) and anti-CD28 (1µg/mL) for 12 hours. Cell samples were collected during the course of the experiment at select time points for gene expression analysis using RT-PCR and for metabolic assays using the XF96 Analyzer.

Establishing the Generation and Kinetics of Th1 and Th2 cells

To ensure that Th1 and Th2 cells were generated under the appropriate skewing conditions and to establish a reference for the kinetics of metabolic gene expression of Th1 and Th2 cells, the gene expression of the key transcription factors of Th1 and Th2 cells, Tbet and Gata3 respectively, was analyzed using RT-PCR. As expected, Tbet was upregulated in Th1 skewed cells and Gata3 gene expression was upregulated in Th2 skewed cells. Tbet and Gata3 expression was observed to be at its highest at 24 hours during induction of Th1 and Th2 cells respectively (**Figure 9**).

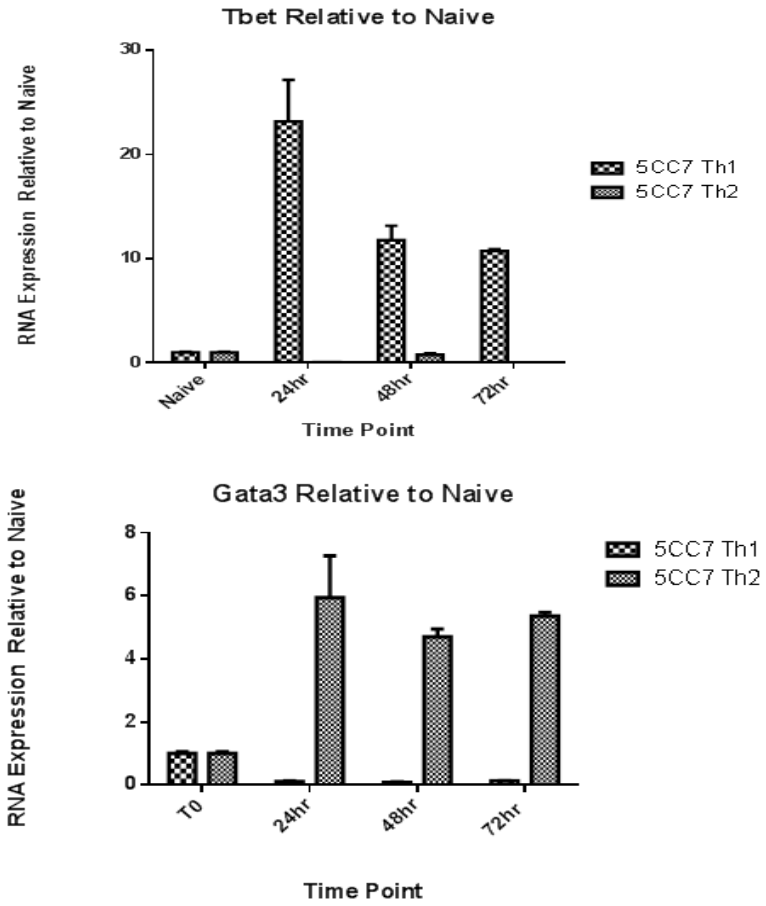

Figure 9. Tbet and Gata3 gene expression in 5C.C7 Th1 and Th2 skewed cells at 24 hours, 48 hours, and 72 hours after stimulation. RNA expression is expressed relative to 5C.C7 naive CD4+ T cells (isolated prior to stimulation).

Metabolism During Induction Phase of Th1 and Th2 cells

Upon demonstrating that Th1 and Th2 cells were generated under the appropriate skewing conditions and establishing a reference for the kinetics of metabolic gene expression in Th1 and Th2 cells, the expression of metabolic genes involved in glycolysis, the pentose phosphate pathway, and fatty acid oxidation during the induction phase was next analyzed. The glucose transporter, GLUT-1 (Slc2a1), glucose-6-phosphate dehydrogenase (G6PD), the rate-controlling enzyme of the pentose phosphate

pathway, and phosphofructokinase-1 (PFK-1), the third enzyme and very important regulatory point of the glycolytic pathway, were all upregulated during the induction phase of both Th1 and Th2 cells, with maximal gene expression occurring around 48 hours and thus, 24 hours after maximal expression of the transcription factors Tbet and Gata3 (**Figure 10**).

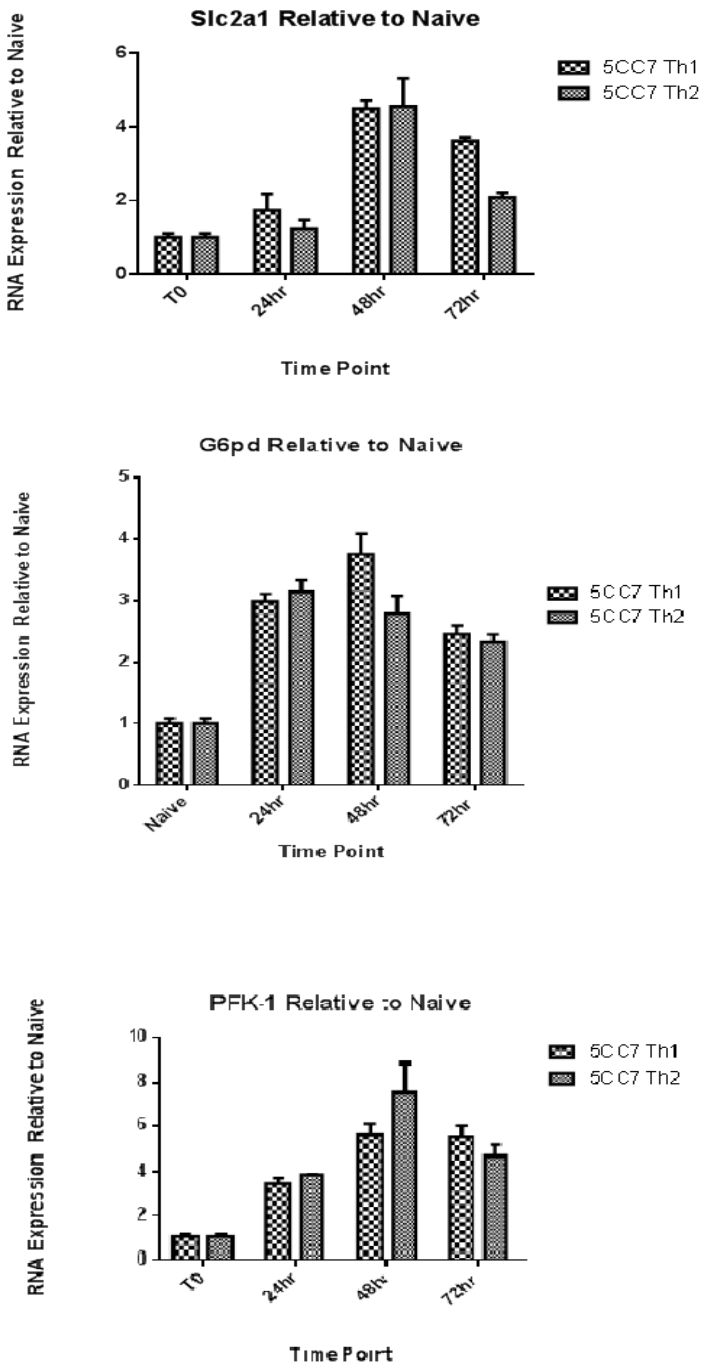

Figure 10. Slc2a1 (GLUT-1), G6PD, and PFK-1 gene expression in 5C.C7 Th1 and Th2 skewed cells at 24 hours, 48 hours, and 72 hours after stimulation. RNA expression is expressed relative to 5C.C7 naive CD4+ T cells (isolated prior to stimulation).

The gene expression of carnitine palmitoyltransferase I (CPT1a), an enzyme essential for fatty acid oxidation,was downregulated during the induction phase of Th1 and Th2 cells at 24 hours, when the highest levels of the transcription factors Tbet and Gata3 were observed in Th1 and Th2 cells respectively. However, CTP1a gene expression increased after the induction phase of Th1 and Th2 cells at 72 hours and the Th2 cells exhibited a greater level of increase in CPT1a gene expression compared to the Th1 cells (**Figure 11**).

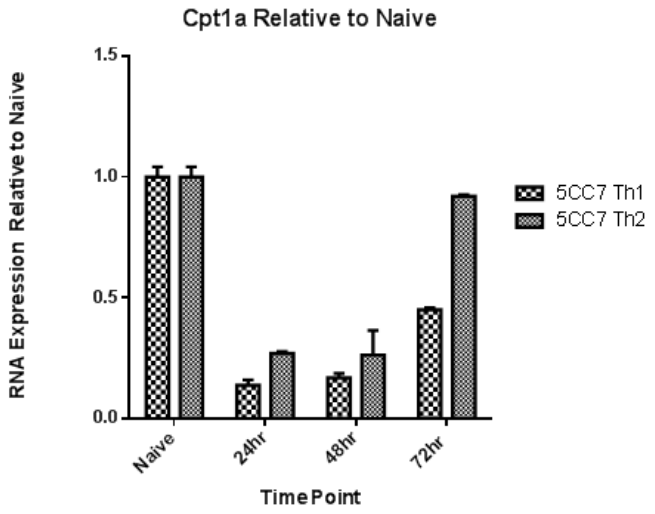

Figure 11. CPT1a gene expression in 5C.C7 Th1 and Th2 skewed cells at 24 hours, 48 hours, and 72 hours after stimulation. RNA expression is expressed relative to 5C.C7 naive CD4+ T cells (isolated prior to stimulation).

In addition to RT-PCR analysis of metabolic gene expression, the extracellular acidification rate (ECAR, a measure of glycolysis) and oxygen consumption rate (OCR, a measure of oxidative phosphorylation) during the induction phase of Th1 and Th2 cells was determined using the XF96 Analyzer. After 24 hours of stimulation, the Th1 skewed cells displayed a higher ECAR and a higher OCR compared to the Th2 skewed cells;

thus, during the induction phase, Th1 skewed cells exhibited an overall higher level of basal metabolism than the Th2 skewed cells.

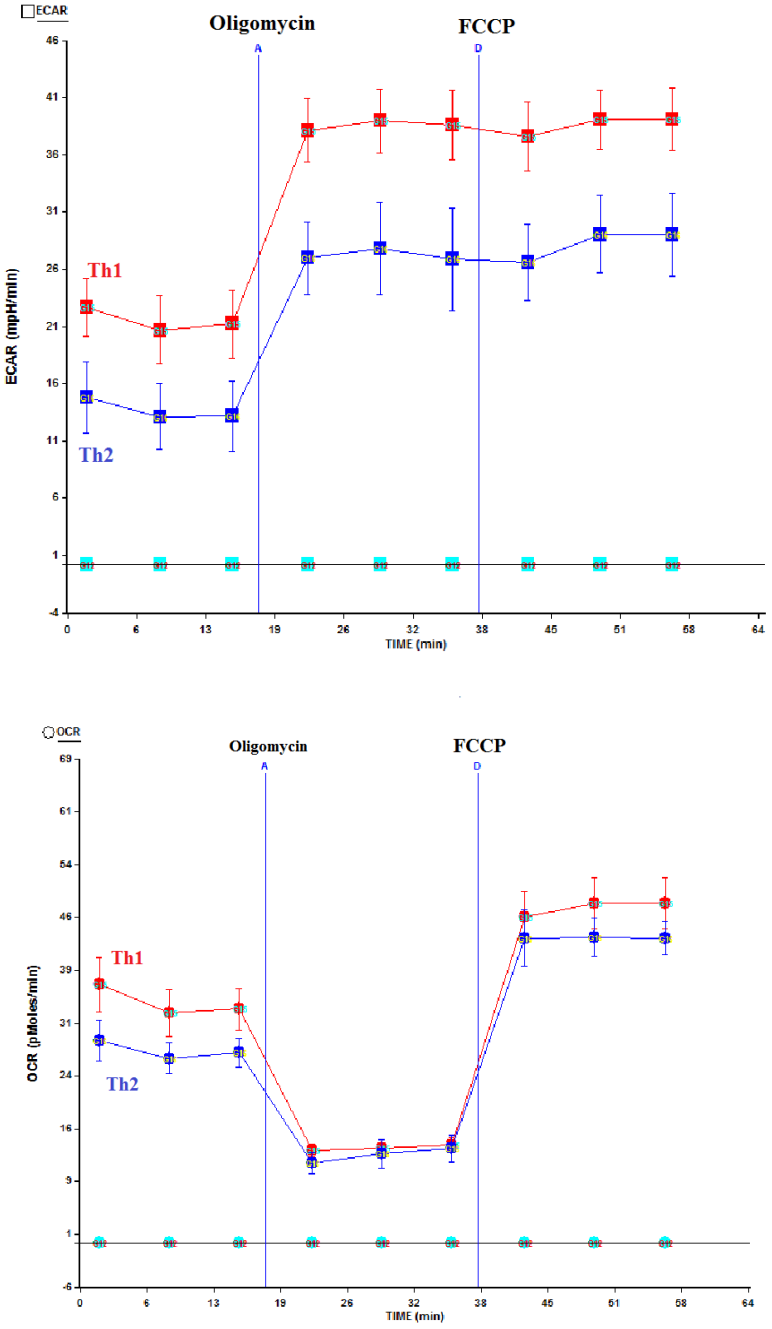

Figure 12. ECAR and OCR of Th1 and Th2 skewed cells at 24 hours after stimulation. Oligomycin, 1 µM of Oligomycin, an ATP synthase inhibitor, was injected into Port A 18 minutes after the start of the experiment and 0.5 µM of FCCP, an ionophore, was injected into Port D an additional 18 minutes later.

Metabolism Upon Rechallenge of Resting Th1 and Th2 cells

Following the 48 hour stimulation and subsequent 5 day expansion, the Th1 and Th2 resting cells were rechallenged for 12 hours using plate-bound anti-CD3 and anti-CD28 in order to next analyze the kinetics of metabolic gene expression upon the restimulation of resting cells. The level of gene expression of the glycolytic enzymes GLUT-1 and PFK-1 increased upon rechallenge of resting Th1 and Th2 cells. Interestingly, it was observed that GLUT-1 and PFK-1 were upregulated to a much greater degree in the rechallenged Th2 cells compared to the rechallenged Th1 cells (**Figure 13**).

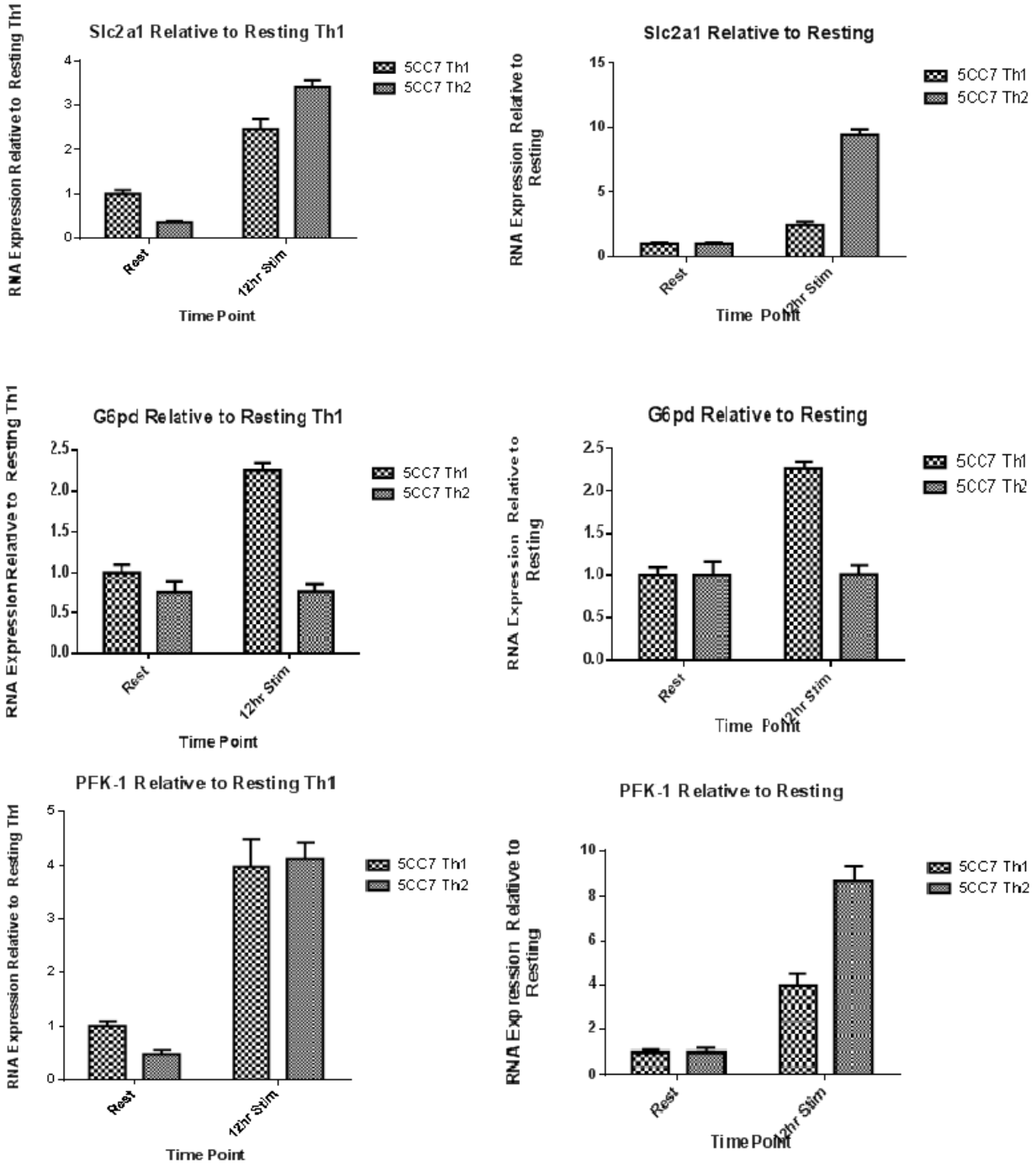

Figure 13. Slc2a1 (GLUT-1), G6PD, and PFK-1 gene expression in 5C.C7 Th1 and Th2 upon rechallenge. RNA expression is expressed relative to resting Th1 cells (left) and relative to the corresponding resting cells (right).

Comparison of the gene expression for the enzyme involved in the committed step of the pentose phosphate pathway, G6PD, in both resting and rechallenged Th1 and Th2 cells provided insightful data. While Th1 cells displayed a higher level of G6PD gene expression upon rechallenge, the level of G6PD gene expression of rechallenged Th2 cells remained relatively the same compared with resting Th2 cells.

Also, CPT1a gene expression, higher in resting Th2 cells than in resting Th1 cells, was downregulated in both Th1 and Th2 cells upon rechallenge. The downregulation of this enzyme was more prominent in Th2 cell (**Figure 14**).

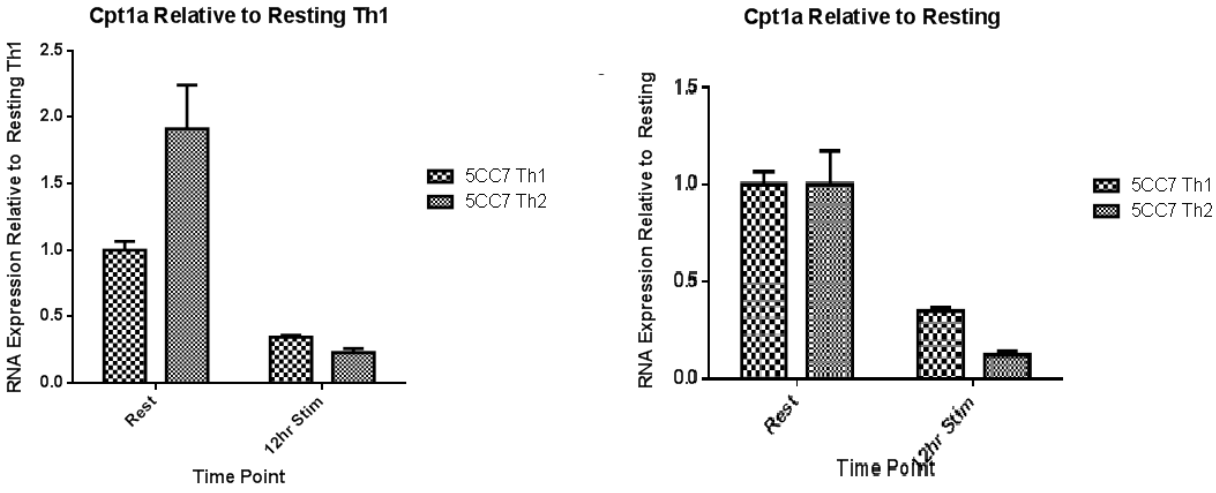

Figure 14. CPT1a gene expression in 5C.C7 Th1 and Th2 upon rechallenge. RNA expression is expressed relative to resting Th1 cells (left) and relative to the corresponding resting cells (right).

Interestingly, the gene expression of the key transcription factor of Th1 cells, Tbet, increased upon the rechallenge of Th1 and Th2 cells and the gene expression of the

key transcription factor of Th2 cells, Gata3, decreased upon rechallenge of Th1 and Th2 cells (**Figure 15**).

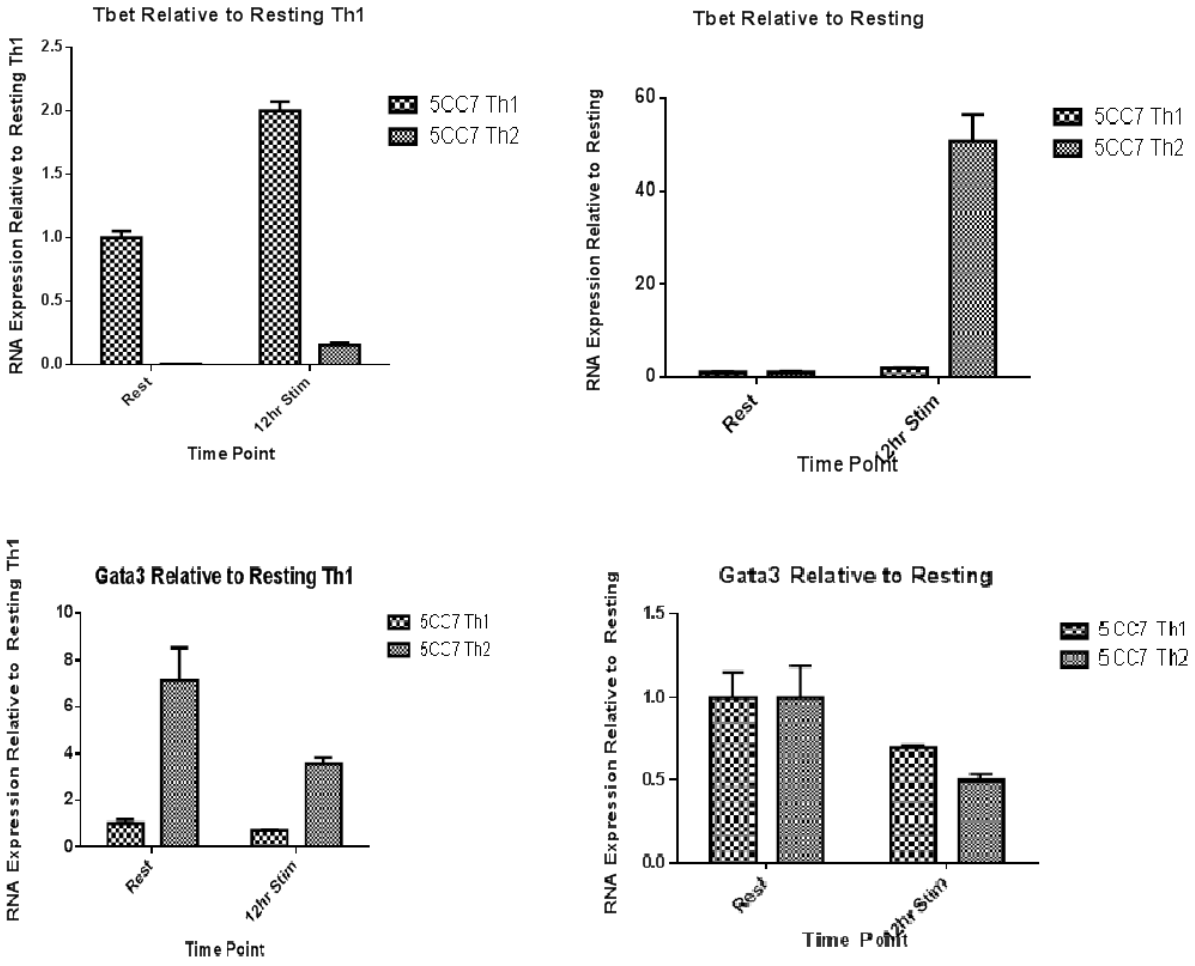

Figure 15. Tbet and Gata3 gene expression in 5C.C7 Th1 and Th2 upon rechallenge. RNA expression is expressed relative to resting Th1 cells (left) and relative to the corresponding resting cells (right).

Furthermore, the extracellular acidification rate and oxygen consumption rate of Th1 and Th2 cells was determined upon rechallenge. After 12 hours of restimulation, the

Th2 cells exhibited higher ECAR and OCR levels compared to the Th1 cells; thus, in contrast to the findings observed during the induction phase, Th2 cells exhibited an overall higher level of basal metabolism than the Th1 cells upon rechallenge.

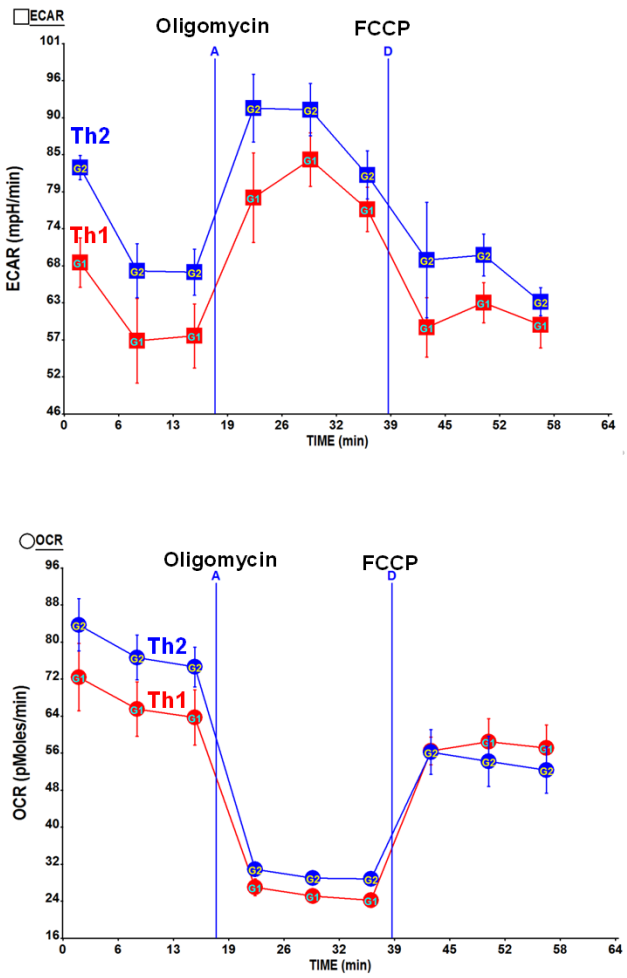

Figure 16. ECAR and OCR of rechallenged Th1 and Th2 cells. 1 µM of Oligomycin, an ATP synthase inhibitor, was injected into Port A 18 minutes after the start of the experiment and 0.5 µM of FCCP, an ionophore, was injected into Port D an additional 18 minutes later.

In addition to using the transgenic 5C.C7 strain, metabolic assays were also performed on CD4+ T cells isolated from the C57BL/6 strain. The CD4+ T cells were stimulated for 48 hours under Th1 and Th2 skewing conditions, and then expanded with IL-2 for 5 days. Following expansion, cells were rechallenged during the Seahorse assay in the XF96 Cell Culture Microplate with a solution consisting of 2 µg/mL of anti-CD3, 2 µg/mL of anti-CD28, and 1 µg/mL of IgG.

Effect of mTOR Kinase Inhibitors Upon Rechallenge of Resting Th1 and Th2 Cells

To determine the effects of mTOR kinase inhibitors on the metabolism of the rechallenged Th1 and Th2 cells, resting Th1 and Th2 cells were either administered rapamycin (a selective mTORC1 inhibitor), PP242 (a mTOR kinase inhibitor), or media and then rechallenged 1 hour later during the Seahorse assay. Surprisingly, the addition of rapamycin had almost no effect on the ECAR levels in both Th1 and Th2 cells. However, a significant decrease in ECAR levels was observed following the addition of PP242 to Th1 cells and Th2 cells (**Figure 17**).

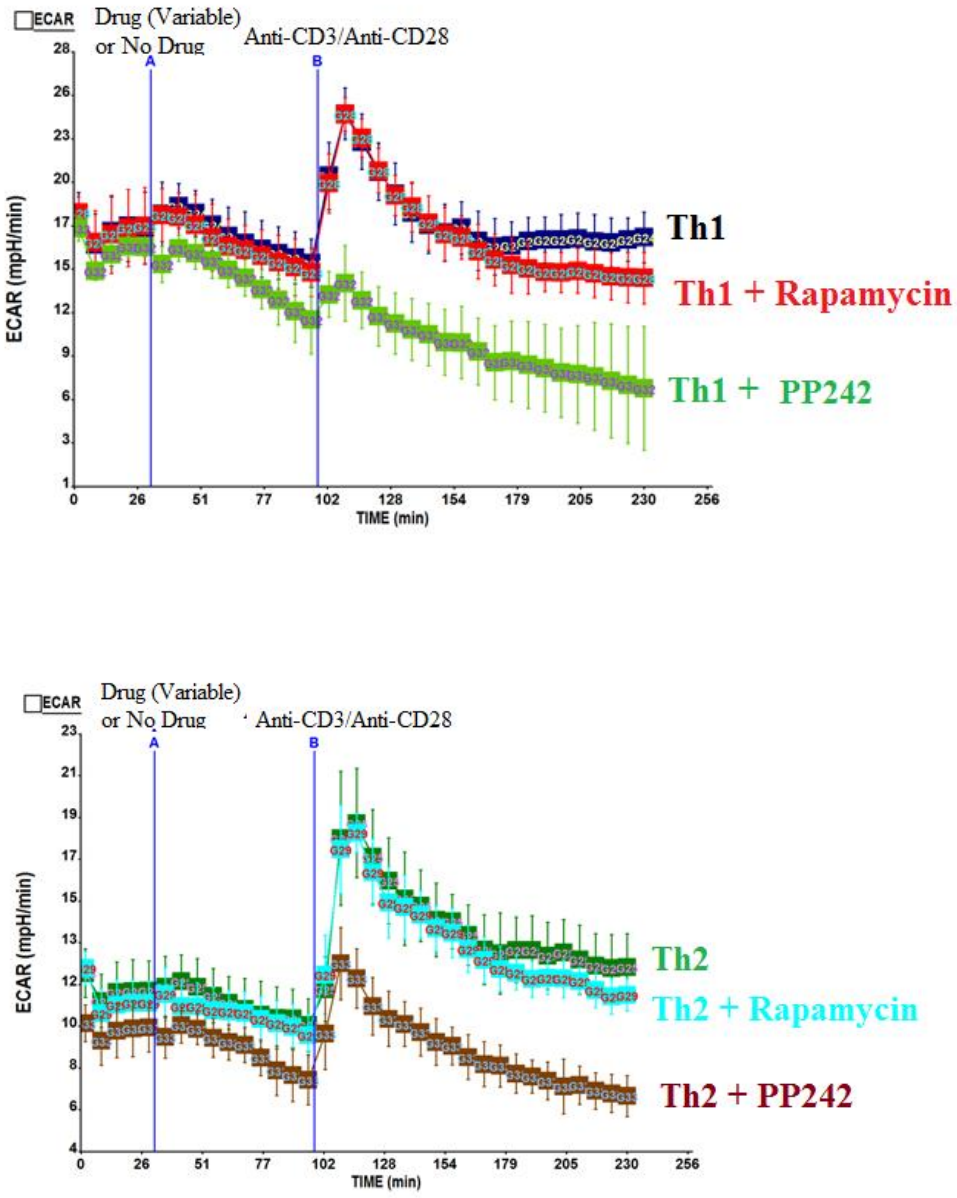

Figure 17. during assay. For each T cell subset, the cells had either 1 µM of Rapamycin, 1 µM of PP242, an mTOR kinase inhibitor, or media injected into Port A 30 minutes after the start of the experiment. All groups of cells were restimulated with 2 µg/mL of anti-CD3, 2 µg/mL of anti-CD28, and 1 µg/mL of IgG approximately 1 hour later.

DISCUSSION

Controlled modulation of the immune system is critical for both the initiation and termination of a proper immune response to a specific type of antigen. Dysregulation of the balance between immunostimulatory and immunosuppressive responses may have deleterious consequences for the host. One of the fundamental goals of immunological research is to elucidate the cellular and molecular mechanisms that regulate the immune system in order to manipulate immune responses in clinical settings such as autoimmunity, allergy, infectious disease, transplantation, and cancer.

The outcome of the immune response is a result of enormously complex interactions between the cells of the immune system and the immune microenvironment. The CD4+ T cells in particular are a subset of lymphocytes involved in cell-mediated immunity that upon encountering their cognate antigen can differentiate into many distinct subsets, each with a specific effector function. The immune microenvironment dictates the outcome of antigen recognition by helping to guide the naive T cell to differentiate into a specific effector cell.

Distinct functions of cells are in part defined by distinct bioenergetic and biosynthetic requirements. The study of the metabolic demands of the particular subsets of T cells and their kinetics has important clinical implications as specific inhibitors of metabolism may be used to direct immune response. The work described herein provides insight into the dynamic changes in the metabolic programs of cells that are associated with activation, commitment to a specific effector lineage, and rechallenge.

The metabolism of CD4+ T cells was examined during the induction phase of Th1 and Th2 cells and also upon the restimulation of resting Th1 and Th2 cells. It was found that during the induction phase of Th1 and Th2 cells, the level of gene expression of the glucose transporter GLUT-1, glucose-6-phosphodehydrogenase (G6PD), the rate-controlling enzyme of the pentose phosphate pathway, and phosphofructokinase-1 (PFK-1), the third enzyme and very important regulatory point of the glycolytic pathway, increased. Also, the enzyme essential for fatty acid oxidation, carnitine palmitoyltransferase 1a (CPT1a), decreased. Thus, since the cell upregulates machinery involved in glycolysis and the pentose phosphate pathway and downregulates machinery involved in fatty acid oxidation, this suggests that upon the activation of a naïve cell, the cell becomes more dependent upon glycolysis and less reliant on fatty acid oxidation for its bioenergetics and biosynthetic needs.

These findings correlate well with the established functions of naïve cells and cells in the incipient stages following activation. A naïve cell's primary role is to migrate through the periphery, seeking its antigen. The metabolic demands of such a cell are relatively low and thus naïve cells may meet all of their bioenergetics and biosynthetic requirements by employing a catabolic program, utilizing fatty acid oxidation, the tricarboxylic acid cycle, and autophagy. A newly activated cell undergoes many rounds of replication within the first 24 hours and expansion requires both energy and *de novo* synthesis of many macromolecules; oxidative glycolysis provides a solution to the heavy metabolic demands placed on the cell since by-products of this pathway may be used as substrates for protein, nucleotide, and lipid biosynthesis. Therefore, it is beneficial for activated T cells to shift to a more glycolytic and anabolic profile.

After the induction phase, around 72 hours, it was found that CPT1a gene expression increased in both Th1 and Th2 cells. Similar to naïve cells, resting cells do not possess the metabolic demands of activated T cells. Therefore, it is not surprising to see an increase in fatty acid oxidation machinery in resting cells. Interestingly, the Th2 cells upregulated Cpt1a to a greater extent than the Th1 cells.

Upon rechallenge, GLUT-1 and PFK-1 expression increased in both Th1 and Th2 cells, though these genes were upregulated to a much greater degree in the rechallenged Th2 cells compared to the rechallenged Th1 cells. In addition, CPT1a gene expression was downregulated in both Th1 and Th2 cells upon rechallenge. The kinetics of the enzyme involved in fatty acid oxidation observed in the transition of resting cells to restimulated cells are similar to those measured during the transition of naïve cells to activated cells. The differences in metabolic gene expression observed in the Th1 and Th2 cells upon rechallenge suggest a more memory-like role for Th2 cells. Resting Th2 cells appear to rely more on fatty acid oxidation and less on glycolysis than Th1 cells. Following reactivation, rechallenged Th2 upregulate glycolytic machinery and downregulate fatty acid oxidation machinery to a greater degree than rechallenged Th1 cells.

Furthermore, while Th1 cells upregulated G6PD gene expression upon rechallenge, the level of G6PD gene expression of rechallenged Th2 cells remained relatively the same compared with resting Th2. Glucose-6-phosphate dehydrogenase is the rate-controlling enzyme of the pentose phosphate pathway. Therefore, it appears that the Th1 cells utilize the pentose phosphate pathway to a greater extent than Th2 cells upon rechallenge.

Finally, as a central signal integrator and regulator of many cellular metabolic programs, mTOR signaling is intimately associated with T cell metabolism. Specific inhibitors of the mTOR signaling pathway may provide crucial clues to the link between mTOR and T cell metabolism. The effects of mTOR kinase inhibitors on the metabolism of the rechallenged Th1 and Th2 cells were determined by measuring the extracellular acidification rate (ECAR, a measure of glycolysis), and the oxygen consumption rate (OCR, a measure of oxidative phosphorylation). It was surprising to observe no significant effect on the extracellular acidification rate upon the addition of rapamycin, a selective mTORC1 inhibitor, on Th1 cells. However, toward the later time points of the metabolic assay, the ECAR levels of Th1 cells seem to be lower than the ECAR levels of the Th2 cells. These findings suggest that, during later time points, the Th1 cells may be affected by rapamycin more than Th2 cells. Future studies extending the time interval between the addition of rapamycin and restimulation and the duration of the experiment may help confirm the differential effect of rapamycin on Th1 and Th2 cells. It is possible that no significant effect of rapamycin on the metabolism of Th1 cells is observed only in the short term since rapamycin's mechanism of action is thought to impact the assembly of the mTORC1 complex. If the mTORC1 machinery is already available in the cell, rapamycin would have little to no effect in the short term. Significant impacts would most likely only be observed in the long term experiments in which assembly of machinery within the cell would be necessary for continued signaling through the mTOR pathway.

In contrast to rapamycin, the addition of PP242, an mTOR kinase inhibitor, drastically decreased the ECAR levels of both cells, suggesting that both Th1 and Th2 cells rely on mTOR for the regulation of their metabolism.

LITERATURE CITED

Abraham RT, Wiederrecht GJ. Immunopharmacology of rapamycin. Annu Rev Immunol. 1996;14:483-510.

Andrade MA, Bork P. HEAT repeats in the Huntington's disease protein. Nat Genet. 1995;11(2):115-6.

Bar-peled L, Schweitzer LD, Zoncu R, Sabatini DM. Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. Cell. 2012;150(6):1196-208.

Bayascas JR, Allessi DR. Regulation of Akt/PKB Ser473 phosphorylation. Mol Cell. 2005; 18(2):143-5

Brunet A, Park J, Tran H, Hu LS, Hemmings BA, Greenberg ME. Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). Mol Cell Biol. 2001; 21(3):952-65.

Chen C, Pore N, Behrooz A, Ismail-beigi F, Maity A. Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. J Biol Chem. 2001;276(12):9519-25.

Chen J, Zheng XF, Brown EJ, Schreiber SL. Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. Proc Natl Acad Sci USA. 1995;92(11):4947-51.

Cote-sierra J, Foucras G, Guo L, et al. Interleukin 2 plays a central role in Th2 differentiation. Proc Natl Acad Sci USA. 2004;101(11):3880-5.

Cui G, Qin X, Wu L, et al. Liver X receptor (LXR) mediates negative regulation of mouse and human Th17 differentiation. J Clin Invest. 2011;121(2):658-70.

Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. Nature. 2007;450(7170):736-40.

Dames SA, Mulet JM, Rathgeb-szabo K, Hall MN, Grzesiek S. The solution structure of the FATC domain of the protein kinase target of rapamycin suggests a role for redox-dependent structural and cellular stability. J Biol Chem. 2005;280(21):20558-64.

Dang EV, Barbi J, Yang HY, et al. Control of T(H)17/T(reg) balance by hypoxia-inducible factor 1. Cell. 2011;146(5):772-84.

Delgoffe GM, Kole TP, Zheng Y, et al. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. Immunity. 2009;30(6):832-44.

Delgoffe GM, Pollizzi KN, Waickman AT, et al. The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. Nat Immunol. 2011;12(4):295-303.

Düvel K, Yecies JL, Menon S, et al. Activation of a metabolic gene regulatory network downstream of mTOR complex 1. Mol Cell. 2010;39(2):171-83.

Forman BM, Chen J, Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc Natl Acad Sci USA. 1997;94(9):4312-7.

Fox CJ, Hammerman PS, Thompson CB. Fuel feeds function: energy metabolism and the T-cell response. Nat Rev Immunol. 2005;5(11):844-52.

Frauwirth KA, Riley JL, Harris MH, et al. The CD28 signaling pathway regulates glucose metabolism. Immunity. 2002;16(6):769-77.

García-martínez JM, Alessi DR. mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). Biochem J. 2008;416(3):375-85.

Gilmour KC, Pine R, Reich NC. Interleukin 2 activates STAT5 transcription factor (mammary gland factor) and specific gene expression in T lymphocytes. Proc Natl Acad Sci USA. 1995;92(23):10772-6.

Guertin DA, Stevens DM, Thoreen CC, et al. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. Dev Cell. 2006;11(6):859-7

Gwinn DM, Shackelford DB, Egan DF, et al. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell. 2008;30(2):214-26.

Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev. 2004;18(16):1926-45.

Ikenoue T, Inoki K, Yang Q, Zhou X, Guan KL. Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. EMBO J. 2008;27(14):1919-31.

Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol. 2002;4(9):648-57.

Jacinto E, Loewith R, Schmidt A, et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol. 2004;6(11):1122-8.

Kim E, Goraksha-hicks P, Li L, Neufeld TP, Guan KL. Regulation of TORC1 by Rag GTPases in nutrient response. Nat Cell Biol. 2008;10(8):935-45.

Kovacina KS, Park GY, Bae SS, et al. Identification of a proline-rich Akt substrate as a 14-3-3 binding partner. J Biol Chem. 2003;278(12):10189-94.

Long X, Lin Y, Ortiz-vega S, Yonezawa K, Avruch J. Rheb binds and regulates the mTOR kinase. Curr Biol. 2005;15(8):702-13.

Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. Nat Rev Mol Cell Biol. 2009;10(5):307-18.

Macintyre AN, Rathmell JC. Activated lymphocytes as a metabolic model for carcinogenesis. Cancer Metab. 2013;1(1):5.

Michalek RD, Gerriets VA, Jacobs SR, et al. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. J Immunol. 2011;186(6):3299-303.

Parry RV, Chemnitz JM, Frauwirth KA, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Mol Cell Biol. 2005;25(21):9543-53.

Powell JD, Pollizzi KN, Heikamp EB, Horton MR. Regulation of immune responses by mTOR. Annu Rev Immunol. 2012;30:39-68.

Qin H, Wang L, Feng T, et al. TGF-beta promotes Th17 cell development through inhibition of SOCS3. J Immunol. 2009;183(1):97-105.

Sancak Y, Peterson TR, Shaul YD, et al. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science. 2008;320(5882):1496-501.

Sarbassov DD, Ali SM, Sengupta S, et al. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol Cell. 2006;22(2):159-68.

Shi LZ, Wang R, Huang G, et al. HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. J Exp Med. 2011;208(7):1367-76.

Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell. 2000;100(6):655-69.

Takahashi T, Hara K, Inoue H, et al. Carboxyl-terminal region conserved among phosphoinositide-kinase-related kinases is indispensable for mTOR function in vivo and in vitro. Genes Cells. 2000;5(9):765-75.

Takeda K, Tanaka T, Shi W, et al. Essential role of Stat6 in IL-4 signalling. Nature. 1996;380(6575):627-30.

Toker A, Newton AC. Cellular signaling: pivoting around PDK-1. Cell. 2000;103(2):185-8.

Van stipdonk MJ, Hardenberg G, Bijker MS, et al. Dynamic programming of CD8+ T lymphocyte responses. Nat Immunol. 2003;4(4):361-5.

Waickman AT, Powell JD. mTOR, metabolism, and the regulation of T-cell differentiation and function. Immunol Rev. 2012;249(1):43-58.

Wang R, Dillon CP, Shi LZ, et al. The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. Immunity. 2011;35(6):871-82.

Weichhart, T. (2012). Mammalian target of rapamycin: a signaling kinase for every aspect of cellular life. In T. Weichhart (Ed.), *mTOR: Methods and Protocols* (Vol. 821, pp. 1-2). New York, NY: Humana Press.

Wu Q, Liu Y, Chen C, et al. The tuberous sclerosis complex-mammalian target of rapamycin pathway maintains the quiescence and survival of naive T cells. J Immunol. 2011;187(3):1106-12.

Yang K, Neale G, Green DR, He W, Chi H. The tumor suppressor Tsc1 enforces quiescence of naive T cells to promote immune homeostasis and function. Nat Immunol. 2011;12(9):888-97.

Yang Q, Guan KL. Expanding mTOR signaling. Cell Res. 2007;17(8):666-81.

Zheng Y, Delgoffe GM, Meyer CF, Chan W, Powell JD. Anergic T cells are metabolically anergic. J Immunol. 2009;183(10):6095-101.

Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). Annu Rev Immunol. 2010;28:445-89.