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The Warburg effect controls cell growth and differentiation by altering N-glycosylation

DISSERTATION

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by

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DEDICATION

To

my parents,

Jay and Kim Klohe, my brother Holdt Klohe, and
Grandmother Marion Araujo

TABLE OF CONTENTS

LIST OF FIGURES

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ABSTRACT OF THE DISSERTATION

The Warburg effect controls cell growth and differentiation by altering N-glycosylation

By

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Doctor of Philosophy in Biomedical Sciences

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Rapidly proliferating cells (T cells, cancer cells) increase glucose uptake yet undergo a metabolic switch that paradoxically shifts energy production from oxidative phosphorylation to the less efficient aerobic glycolysis, a poorly understood phenomenon known as the Warburg effect. This reduction in energy efficiency markedly increases glucose utilization and has been rationalized as enhanced requirements for glucose metabolites in biomass generation. Recent data in T cells suggests a functional role for the Warburg effect in IFNγ production and the differentiation of T helper 17 (T_H17) versus T regulatory (Treg) cells. Here we report that a primary function of the Warburg effect in T cells is to limit glucose metabolite supply to Golgi N-glycosylation. Branched N-glycans regulate the concentration and signaling of surface glycoproteins to control T cell development and function. N-glycan branching depends on UDP-GlcNAc biosynthesis via the hexosamine pathway, a metabolic pathway that competes with glycolysis for glucose-derived fructose-6-phosphate. In glycolytic T cells, the hexosamine pathway is starved of fructose-6-phosphate, reducing *de novo* UDP-GlcNAc biosynthesis and N-glycan branching. This drives growth and pro-autoimmune

T_H17 over anti-autoimmune induced T regulatory cell differentiation by reducing IL-2 receptor-α (CD25) surface retention and signaling; effects reversed by restoring UDP-GlcNAc production via salvage with N-acetylglucosamine or by over-expression of *Mgat5*. Restoring UDP-GlcNAc production downstream of fructose-6-phosphate has no impact on the glycolytic state of the cell, while forcing oxidative phosphorylation raises branching but does not alter T cell differentiation if branching is inhibited. Our data suggest that the Warburg effect primarily functions to control cell growth and differentiation by controlling glucose flux into the hexosamine pathway and N-glycan branching. These results address a long-standing mystery of the Warburg effect and have widespread implications for immunity, autoimmunity and cancer.

Chapter 1

Introduction

In the presence of pathogens, activation of antigen-specific T cells results in increased glucose uptake and paradoxically, a metabolic shift from the energy efficient oxidative phosphorylation to the less efficient glycolysis even in the presence of oxygen. This phenomenon is also observed in cancer cells and is known as the Warburg effect [1]. The reliance on glycolysis to generate ATP when oxygen is readily available for oxidative phosphorylation is incompletely understood but has been attributed to the need for increased production of biosynthetic building blocks required for replication and growth [2-4]. Recent data suggests that the switch to glycolytic metabolism by T cells may play a functional role in cell fate decisions [5, 6].

Asn (N) linked glycosylation plays a critical role in T cell growth and differentiation and requires glucose and glucose derived sugars for biosynthesis. N-glycan branching depends on UDP-GlcNAc biosynthesis via the hexosamine pathway, which is a competitor of glycolysis for glucose derived fructose-6-phosphate. Branched N-glycans bind galectins at the cell surface, forming a macromolecular lattice that regulates the concentration and signaling of multiple surface receptors. The galectin-glycoprotein lattice is increasingly recognized as a major regulatory mechanism of T cell fate determination. Here we investigate the hypothesis that the Warburg effect regulates N-glycan branching to control T cell growth and differentiation.

The N-glycosylation pathway

Eukaryotic cell surfaces are dominated by a dense coating of sugars or glycans which defines the glycocalyx, an ~100 nanometer wide macromolecular structure consisting of glycans attached to proteins and lipids. Extensive diversity in the

composition and function of the carbohydrate moieties is a common theme in protein glycosylation [7]. The plethora of structures, size, and complexity of glycans suggest a significant role for information encoding distinct from protein and nucleic acid biosynthesis [8].

The majority of cell surface receptors and transporters are co-translationally modified with asparagine (N)-linked glycans, with the number of N-glycans per glycoprotein genetically encoded. The enzyme Oligosaccharyl transferase initiates N-glycan synthesis by transferring the preassembled $Glc_3Man_9GlcNAc_2$ structure to the asparagine residue of the Asn-X-Ser/Thr (NXS/T, X not Proline) motif of proteins in the endoplasmic reticulum (ER) [9]. This then gets modified following a sequential but incomplete action of a series of glycohydrolases and glycosyltransferases [10, 11]. The 3 glucoses of the newly added glycan are removed in the ER by Glucosidase I and Glucosidase II immediately after being transferred to the protein [9]. Mannosidase I then removes four mannose residues from the trimmed glycan to then generate $Man_5GlcNAc_2$, which is the substrate of the first N-acetylglucosaminyltransferase (**Fig. 1.1**) [12].

N-acetylglucosaminyltransferase I, II, IV, and V, encoded by the genes *Mgat 1*, *-2*, *-4a/b*, and *-5*, act sequentially to transfer N-acetyl-D-glucosamine (GlcNAc) from the common sugar nucleotide substrate UDP-GlcNAc to N-glycan intermediates, but with declining efficiency. These branches are then extended with galactose residues which are added on to the GlcNAc residues by Galactosyltransferase-3, producing mono, bi-, tri-, and tetra-antennary N-acetyllactosamine branched N-Glycans (**Fig. 1.1**) [10, 11]. N-acetyllactosamine can be repeatedly extended to form poly-N-acetyllactosamine until

sialic acid is added, terminating further N-acetyllactosamine additions [13, 14]. Thus, the same protein can have many different glycoforms dependent upon the availability of the substrate UDP-GlcNAc and expression and activity of the enzymes responsible for the modifications.

N-glycan branching deficiency

The branching enzymes form a linear pathway and require the initiation of N-glycan branching by Mgat1, the first branching enzyme in the pathway for all subsequent branches. Various groups have studied the biological significance of the enzymes in this pathway through chemical inhibition and transgenic mouse models. For example swainsonine, a chemical inhibitor of α-mannosidase II, acts immediately downstream of Mgat1 to block N-glycan branching at the mono-antennary stage. However, chemical inhibition of mannosidase II only provides a partial reduction in branching and may result in positive feedback to restore N-acetyllactosamine content via poly-lactosamine extension. In contrast, the inhibitor kifunensine acts immediately upstream of Mgat1 to inhibit mannosidase I and all branching.

Transgenic mouse models have also been widely used to further characterize N-glycan branching deficiencies. *Mgat1* deficiency results in embryonic lethality, with defects in the vasculature, neural tube, and in the determination of heart loop asymmetry [15, 16]. Contrarily, *Mgat1*$^{+/-}$ embryos develop normally and adult mice are indistinguishable from their wild-type littermates. Loss of *Mgat2* results in a modest degree of embryonic lethality as well as peri-natal mortality, with most live *Mgat2*-null mice perishing within the first week of postembryonic life [17]. *Mgat2* deficiency models

the human disease caused by mutational inactivation of the human *MGAT2* locus known as Congenital Disorders of Glycosylation type IIa (CDG-IIa) [18, 19]. *Mgat4* has two isoforms, *Mgat4a* and *Mgat4b*. It has been shown that the mouse *Mgat4a* gene is selectively expressed, with dominance in the pancreas [20]. It is required for Glut-2 residency on the β cell surface and loss of *Mgat4a* leads to the induction of Type II Diabetes [20]. *Mgat5* deficient mice lack the tri- and tetra-antennary β1,6 GlcNAc branched structures, and display late onset spontaneous autoimmune disease, T cell hypersensitivity, enhanced agonist induced T cell receptor (TCR) clustering, and enhanced proliferation [21].

The galectin-glycoprotein lattice

Galectins are a 15-member family of carbohydrate binding proteins in mammals that possess two or more glycan-binding sites as monomers and/or multimers. N-acetyllactosamine is the minimal binding unit for galectins, which are ubiquitously expressed at the cell surface and extracellular matrix and interact with multivalent glycan ligands to form lattices [22-24]. Galectins are also expressed in the cytoplasm where they may have unique effects distinct from cell surface glycoprotein interactions [25]. Galectins are classified into three structural groups; namely proto-type, tandem, and chimeric galectins [26]. Proto-type galectins (galectin-1, -2,-5,-7,-10,-11,-13,-14,-15) possess a single carbohydrate recognition domain (CRD) and frequently dimerize [22]. Tandem repeat galectins (galectin -4, -6, -8, -9, and -12) contain two CRDs connected by a short linker region that may also dimerize [27]. Chimeric galectins

(galectin-3) contain a single CRD connected to an N-terminal region that allows formation of pentamers in the presence of multivalent ligand [22].

The binding of galectins to their N-glycan ligands regulates glycoprotein distribution and concentration at the plasma membrane via formation of a macro-molecular galectin-glycoprotein lattice [28]. Binding avidity for individual glycoproteins increases in proportion to the number of N-acetyllactosamine units per N-glycan and the number of N-glycans per protein [28]. Thus, alterations in Golgi production of N-acetyllactosamine ligands is expected to alter galectin-glycoprotein interactions.

The galectin-glycoprotein lattice regulates receptor function at the cell surface by controlling the clustering/lateral movement of receptors, membrane localization and cell surface retention/endocytosis rates [8, 24, 28-30]. Growth promoting receptors such as the TCR and receptor tyrosine kinases frequently have a high density of N-glycans per protein molecule (i.e. five or more N-glycans). Contrarily, growth inhibitory receptors such as transforming growth factor-β receptors I and II (TβR) and cytotoxic T-lymphocyte antigen-4 (CTLA-4) possess few N-glycan sites (i.e. four or less). Therefore, low Golgi activity is predicted to result in only positive regulators of growth to be maintained on the cell surface, resulting in a growth promoting state. This is due to the higher density of N-glycans found on these proteins resulting in an enhanced avidity to galectins at low levels of branching. However, with enhanced N-acetyllactosamine content that accompanies branching, the negative regulators of cell growth are able to become incorporated into the galectin-glycoprotein lattice. The lower level of N-glycan sites on these proteins requires a higher level of branching activity in the Golgi to enhance cell surface residency and oppose endocytic loss of receptors. Thus, Golgi

activity has the potential to regulate cell growth characteristics by controlling the transitions from growth to arrest/differentiation [8, 28].

Regulation of T cell growth by N-glycosylation

The T-cell immune response is regulated by an intricate interplay between both stimulatory and inhibitory signals. T cell activation requires the clustering of a threshold number of TCRs at the immunological synapse determined by engagement of the TCR with a specific peptide with major histocompatibility complex (MHC) displayed on the cell surface of an antigen presenting cell (APC) and co-signals via CD28. The galectin-glycoprotein lattice negatively regulates T cell activation by the binding of galectins to N-glycans of the TCR complex, thereby limiting lateral movement and clustering in response to ligands. This suggests that in order to induce clustering and T cell activation the peptide-MHC complex needs to overcome the galectin-TCR interactions and the repulsive force of the lattice. For example, experiments in which defined N-glycosylation sites were removed in the TCR constant domain as well as *Mgat5* deficiency both reduce the avidity of TCR for galectins and enhance avidity for peptide-MHC [31] and anti-CD3 antibody [24]. Self peptide-MHCs are usually unable to activate T cells; however it is possible that the weakening of the galectin-glycoprotein lattice by reductions in N-glycosylation may convert these into activating complexes resulting in a loss of self tolerance. Indeed, a recent study from the Demetriou Lab found that targeted deficiency of *Mgat1* in T cells (*Mgat1*$^{f/f}$ Lck-Cre^{+}) results in a complete lack of peripheral T cells due a profound defect in thymocyte selection. Loss of branching reduces surface expression of CD4 and CD8α in double positive (DP) and single

positive (SP) due to enhanced endocytosis [32]. The CD4 and CD8 co-receptors bind lymphocyte-specific protein tyrosine kinase (Lck) and drive TCR responses to low affinity peptide-MHC and therefore promote positive selection by augmenting recruitment of Lck to TCR [33-36]. Reduced CD4 and CD8 surface retention from *Mgat1* deficiency decreases delivery of Lck to TCR, thereby losing sensitivity of low affinity TCR for peptide-MHC and resulting in death by neglect [32]. With high affinity peptides, loss of branching enhances TCR clustering and calcium signaling, leading to death by negative selection. Thus, N-glycan branching defines the TCR affinity for peptide-MHC that leads to positive selection and production of mature T cells [32].

Mgat5 deficient mice display various autoimmune defects including spontaneous autoimmune-mediated glomerulonephritiis, enhanced susceptibility to delayed-type hypersensitivity, and experimental autoimmune encephalomyelitis (EAE), the latter a model for multiple sclerosis (MS) [24]. Therefore the galectin-glycoprotein lattice appears to set an activation threshold at the immunological synapse, and changes in the strength of the lattice have the potential to convert non-activating complexes into agonists.

Following T-cell activation, the cell undergoes multiple rounds of cell division, followed by growth arrest, which is marked by the expression of CTLA-4 at the cell surface 4-5 days after activation [37]. The regulation of the T cell response by inhibitory receptors is critical for T-cell tolerance and autoimmune pathogenesis. CTLA-4 and CD28 are receptors that compete for the CD80/86 co-stimulatory ligand expressed on APCs to negatively or positively regulate T cell proliferation respectively. CD28 is constitutively expressed on the T cell surface in activated and resting cells, whereas

CTLA-4 is predominantly found in endosomes/lysosomes due to constitutive endocytosis. The affinity of CTLA-4 to CD80/86 exceeds that of CD28 and small amounts of CTLA-4 can lead to growth arrest. *CTLA-4* deficiency in mice results in lymphoproliferative disorder and death around 4 weeks of age due to loss of immune tolerance and inflammatory destruction of multiple organs [38].

Human CTLA-4 has only two N-glycan sites suggesting that the surface retention of this receptor by the galectin-glycoprotein lattice should be highly sensitive to changes in N-acetyllactosamine production by the Golgi. Indeed in mice, surface CTLA-4 was lower on *Mgat5* $^{-/-}$ and *Mgat5* $^{+/-}$ than *Mgat5* $^{+/+}$ mouse T cells when stimulated with anti-CD3 [28]. TCR signaling up-regulates *Mgat5* gene expression and N-acetyllactosamine content in N-glycans following activation of naïve T cells to promotes CTLA-4 surface retention [28]. Galectin binding to N-glycans attached to CTLA-4 enhances surface retention by opposing endocytic loss, thereby promoting growth arrest.

Following growth arrest activated $CD4^+$ T cells differentiate into effector T cells based on induction or activation of transcription factors in response to signals from the TCR and cytokines secreted by the activated innate immune system. Lineage commitment is dependent on the integration of actions of cytokine-induced signal transducer and activation of transcription (STAT) factors as well as master transcriptional regulators. T helper 1 (T_H1) effector cells are defined by the expression of the master regulator T-bet, and T_H2 cells by GATA3. T helper subsets are largely defined by cytokine phenotypes. T_H1 cells produce IFN-γ and control infections with intracellular microbes and T_H2 cells secrete IL-4, IL-5, and IL-13 and orchestrate the clearance of extracellular pathogens [39].

The relative balance of T_H1 versus T_H2 can influence susceptibility to autoimmune disease, allergic responses and tumor surveillance. Experiments performed by Bottomly and colleagues [40, 41] demonstrate that enhanced TCR signal strength drives T_H1 differentiation over T_H2. Consistent with these results, *Mgat5*$^{-/-}$ CD4$^+$ T cells display hyperactive TCR signaling and are associated with enhanced IFN-γ production over IL-4 compared to *Mgat5*$^{+/+}$ cells [21]. Similarly, treating human T cells with the mannosidase II inhibitor swainsonine to lower branching also increased IFN-γ production induced by TCR stimulation [21].

Galectin-9 has also been shown to regulate differentiation between effector T cell subsets. Galectin-9 is expressed ubiquitously in different mouse and human cells and tissues [42] and is localized to the cell membrane, cytoplasm, and nucleus [43]. Studies have shown Galectin-9 to be a versatile immunomodulatory molecule with a wide variety of biological activities including cell adhesion, migration, proliferation, apoptosis, interaction with microbial pathogens, Dendritic Cell (DC) maturation, and anti-microbial immunity [44-49]. Due to its wide spread expression on host cells, the biological effects of this lectin are exerted by multiple receptors with distinct effects such as T cell immunoglobulin and mucin containing molecule (Tim-3) [50], cell surface protein disulfide isomerase (PDI) [51], and Immunoglobulin E (IgE) [52]. Previous studies have found Gal-9 is a negative regulator of T_H1 differentiation by binding to Tim-3 and inducing T cell apoptosis and exhaustion [44, 50, 53]. Additionally, the ligation of Gal-9: Tim3 induces intracellular Ca^{2+} flux and activates apoptosis via caspase-1 [50, 54, 55].

Together, these data highlight the fundamental importance of the galectin-glycoprotein lattice in the regulation of T cell responses, such as T cell survival,

proliferation, differentiation, and apoptosis. In particular, further understanding of the mechanisms of differentiation into effector subsets manifest therapeutic opportunities to target inflammation and autoimmunity.

The hexosamine pathway regulates N-glycan branching

The Mgat enzymes share a common substrate, UDP-GlcNAc. β1,6GlcNAc-branched N-glycan expression in T cells is dependent on metabolite supply to UDP-GlcNAc biosynthesis (hexosamine pathway) and in turn, to Golgi Mgat1, -2,-4, and -5. Therefore, increasing availability of substrate (*i.e.* UDP-GlcNAc) to Golgi enzymes by metabolic supplementation *in vitro* with the simple sugar N-acetylglucosamine (GlcNAc) enhances branching. The uptake of GlcNAc into the cell is by macropinocytosis, which is a process that is dependent upon the rate of membrane turnover. Rapidly proliferating cells display a high amount of membrane turnover whereas resting T cells have minimal. Thus, GlcNAc more effectively enhances N-glycan branching in rapidly dividing T cell blasts [56]. Furthermore, it has been shown that treating mice with GlcNAc supplementation in water at the second day of clinical disease attenuated the clinical severity of myelin oligodendrocyte glycoprotein (MOG)-induced EAE, and inhibits T_H1 responses [56]. These data suggest that for treatment of EAE and MS, myelin-activated T cell blasts displaying enhanced macropinocytosis should be more responsive to GlcNAc than resting T cells. This provides a degree of specificity for disease causal cells.

The molecular mechanism underlying the therapeutic effect of GlcNAc has been studied previously [8, 28, 57]. It has been found that GlcNAc mediated inhibition of TCR

signaling, T_H1 differentiation, T cell proliferation, and CTLA-4 endocytosis are reversed by the addition of Golgi branching inhibitors that block N-glycan branching (*i.e.* swainsonine) [57]; confirming that GlcNAc alterations in T cell function are dependent on enhanced N-glycan branching. Furthermore, GlcNAc can be used alone or with other immunoregulatory drugs as it has been used as a dietary supplement for years without reported adverse effects.

T_H17 and iTreg differentiation

$CD4^+$ T regulatory cells (Tregs) play a central role in maintaining immune homeostasis and tolerance to self-antigen [58, 59]. They have the capability to suppress the activation of other T cells in a contact dependent manner [60-62] and are comprised of several subsets, including naturally occurring Treg (nTreg) cells, which are thymically derived, and the induced Treg (iTreg) which are generated in the periphery. Tregs are characterized by expression of the forkhead family transcription factor, forkhead box P3 (Foxp3) and CD25 [60-62]. The role of Foxp3 in the development and function of Treg cells has been elucidated from mutations in Foxp3, which result in fatal autoimmune lymphoproliferative disease. For example, the spontaneous *scurfy* mutation in mice has shown to be a loss of function mutation in the *Foxp3* gene, resulting in death at 3-4 weeks of age and total loss of Tregs [63, 64]. Similarly, humans with IPEX (Immune dysfunction/Polyendocrinopathy/Enteropathy/X-linked) syndrome have mutations in *FOXP3* which results in dysfunctional regulatory T cells and subsequently, autoimmunity [65, 66].

It has been shown that nTreg cells develop in a highly controlled microenvironment [67] whereas iTregs are generated from more varied conditions. iTreg cells appear in the mesenteric lymph nodes during induction of oral tolerance, [68, 69]. In the lamina propria of the gut, iTregs may continuously differentiate in response to microbiota and food antigens [70]. They are also generated in chronically inflamed tissues [71], tumors [72], and transplanted tissues [73].

The generation of iTregs from naive T cells in the periphery or *in vitro,* is dependent on stimulation through the TCR in the presence of TGFβ and IL-2 [74]. Cultures of naïve $CD4^+$ T cells stimulated with anti-CD3 and TGFβ have shown that IL-2 appears to be essential for iTreg cell generation by releasing the TGFβ mediated inhibition of proliferation [74]. Furthermore, experiments utilizing IL-2 neutralization and IL-2 deficient T cells demonstrated the requirement of IL-2 *in vitro* for TGFβ induction of Foxp3 expression and suppressor activity [75]. The mechanism in which TGFβ induces Foxp3 expression is dependent on transcription factors STAT3 and nuclear factor of activated T-cells (NFAT) cooperation at a *Foxp3* gene enhancer element [67, 76]. IL-2 signaling activates STAT5, which binds to the *Foxp3* gene and may cooperate with STAT3 for Foxp3 induction [77, 78].

Contrary to the essential role of TGFβ in iTreg induction, the role of TGFβ in the generation of nTreg cells is less clear. Studies using a T cell specific deletion of TβRII reported that TGFβ was not involved in thymic nTreg cell development ([79-81] and young TGFβ1 deficient mice have a normal number of nTreg cells. Furthermore, other studies have investigated the role of IL-2 in nTreg development and found that IL-

2 deficient and IL-2Rα CD25 deficient mice had normal nTreg levels in the thymus [82, 83].

Activation of auto-reactive T cells causes various autoimmune diseases, which are regulated by peripheral tolerance mechanisms such as Treg cells limiting effector T cell proliferation. For example, MS is an inflammatory demyelinating disease of the central nervous system (CNS), in which uncontrolled auto reactive T cells infiltrate the CNS and attack the myelin sheath. The auto reactive T cells break down the blood brain barrier, gaining access to the CNS where they cause demyelination and inflammation. MS affects more than 2.5 million people worldwide and is divided into relapsing remitting (RR), primary progressive (PP), and secondary progressive subtypes [84].

EAE, the principal animal model of MS, has been widely used to investigate the T effector cells that mediate dysregulated immune function in the CNS. Although T_H1 cells were considered to be the pathogenic effector cells responsible for neuroinflammation, it has been shown that T_H17 cells also play a critical role in the pathogenesis of MS [39]. Pro-inflammatory $CD4^+$ T_H17 cells require the expression of the transcription factor RORγt for development [85] and share a common link with iTreg cells through TGFβ for differentiation into their specific subtypes. Naïve $CD4^+$ T cells can differentiate into T_H17 cells by stimulation through the TCR in the presence of TGFβ and IL-6 [86]. TGFβ can induce both Foxp3 and RORγt expression however this leads to Treg differentiation due to the ability of Foxp3 to associate with RORγt and inhibit RORγt transcriptional activation [87]. However, In the presence of IL-6 this inhibition is abrogated and T_H17 differentiation is initiated [86, 88].

T_H17 cells are defined by the expression of the cytokines IL-17A, IL-17F, and IL-22 [89-91]. IL-17 is a pleiotropic cytokine, which mediates tissue inflammation by inducing many pro-inflammatory cytokines and chemokines [92, 93]. IL-17 is also involved in bacterial immunity through recruitment and activation of macrophages and neutrophils [94]. IL-17 receptor-deficient mice are susceptible to bacterial pneumonias and are unable to form abdominal abscesses [94]. Furthermore, IL-17 over expression has been found in a variety of autoimmune and inflammatory disorders in both mice and humans [95].

In the absence of TGFβ, a combination of pro-inflammatory cytokines IL-1β, IL-6, and IL-23 induce T_H17 differentiation and promote the pathogenesis of EAE [96]. IL-23 has been thought to be required for the generation of T_H17 cells [97, 98] in addition to that of IL-6, however it has been subsequently shown that IL-23R is not expressed on naïve T cells and instead IL-23 acts as a survival signal and expands and maintains this lineage [99]. IL-23 has also been shown to induce the expression of the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) by T_H17 cells which in turn enhances IL-23 production by DCs [100, 101]. Therefore, the IL-23-T_H17-GM-CSF circuit drives T_H17 responses and *de novo* generation of pathogenic T_H17 cells, indicating that the complex interactions between cytokines, and inflammatory factors dictates T_H17 pathogenicity and disease outcomes.

Disease susceptibility to MS is driven by multiple factors such as genetics and environment. How environmental factors contribute to these processes remains less understood. Recent studies have reported that a high salt diet is an environmental risk factor in the development of EAE through enhancing T_H17 induction and EAE

pathogenicity in an IL-23R signaling dependent manner [102, 103]. Kleinewietfeld et al. reported that high concentrations of sodium chloride markedly enhance T_H17 induction along with other molecules of T_H17 cells, including IL-17A, IL-17F, RORγt, and IL-23R as well as GM-CSF and chemokine receptor 6 (CCR6) which mediate enhanced pathogenicity of T_H17 [103]. Furthermore, mice with a high-salt diet exhibit accelerated onset and severity of EAE [103]. Increases in NaCl concentration activate the p38/MAPK pathway to enhance the expression of serum/ glucocorticoid-regulated kinase 1 (SGK1) and nuclear factor of activated T cells 5 (NFAT5), both of which contribute to T_H17 cell differentiation, although whether p38 MAPK signaling in T cells directly regulates T_H17 cell generation is unclear [104]. Moreover, loss of SGK1 in T cells protects the mice from high salt mediated exacerbation of EAE [102].These studies suggest a pathogenic effect of a high salt diet and targeting SGK1 or related molecular pathways may have beneficial effects in MS patients.

The reciprocal relationship between T_H17 and iTreg cell differentiation has been studied by multiple groups. It has been found that exogenously added IL-2 negatively regulates T_H17 differentiation and switches the cell fate to iTreg, an effect that is dependent on STAT5 [105]. In addition, retinoic acid has been shown to suppress T_H17 induction and promote $Foxp3^+$ Treg cell differentiation under T_H17 inducing conditions [106-108]. This was realized following the observation that DCs isolated from the small intestine and mesenteric lymph nodes produce both TGFβ and Retinoic Acid and efficiently mediate the differentiation of naïve T cells into $Foxp3^+$ Treg cells [68, 70].

Tim3 expression is also found on T_H17 cells and Gal-9 binding with Tim3 results in apoptosis of T_H17 differentiated cells [109]. Additionally, Gal-9 suppresses IL-17

mRNA induction of T_H17 cells in the presence of TGFβ and IL-6 while up-regulating Foxp3 expression [109]. However, other data suggests that instead of interacting with Tim3, the interaction of Gal-9 with CD44 and TβR1 clusters the receptors and amplifies TGFβ signaling to induce Foxp3 and Gal-9 expression in a positive feed forward loop involving Smad activation [110]. Gal-9 and CD44 interactions have been shown to drive Foxp3 expression and iTreg differentiation [110].

These data suggest that the differentiation of a naïve $CD4^+$ T cell into a T_H17 or iTreg is dependent upon the environment in which activation occurs and the balance between Foxp3 and RORγt. However, aside from the regulation of differentiation into effector T cell subsets by cytokine signaling, emerging evidence highlights the role of T cell metabolism as an important determinant in T cell fate decisions and functions.

Metabolic regulation of T_H17 and iTreg cell differentiation

Although rapidly proliferating T cells undergo a metabolic shift to aerobic glycolysis, they can still use oxidative phosphorylation (OXPHOS) as a source of energy. It has been found that cells grown in galactose are forced to respire and do not use aerobic glycolysis [111-114]. T cells cultured in galactose activate, generate ATP, and proliferate although at a slower initial rate than those cultured in glucose suggesting that metabolism may play a critical role in pathways other than those driving proliferation [6].

The mechanistic target of Rapamycin (mTOR) and Amp-activated protein kinase (AMPK) have been found to play critical roles in both metabolism and immunity. T cell activation drives mTOR to enhance the expression of glycolytic genes and inhibit lipid

oxidation [115], which is also critical for T effector cells such as T_H1, T_H2, and T_H17 lineages as shown by elevated surface expression of the glucose transporter Glut1 compared with iTreg cells [5, 116]. In contrast, AMPK pathways have been found to inhibit mTOR by suppression of signaling and to promote mitochondrial oxidative metabolism rather than glycolysis [117]. Treg cells are reported to have activated AMPK kinase, and this signaling is sufficient to decrease Glut1 expression [116]. However, iTreg cells aren't metabolically inactive and do exhibit enhanced lipid oxidation compared to other $CD4^+$ subsets [116]. Thus $CD4^+$ Treg and T effector cells use distinct metabolic pathways and metabolism is a fundamental determinant of cell fate.

A key transcription factor for orchestrating the expression of glycolytic enzymes is HIF1 (Hypoxia Inducible Factor 1), which consists of two subunits: the α subunit, which is located in the cytoplasm (HIF1α) and the β subunit which is located in the nucleus (HIF1β) [118]. Under normoxic conditions HIF1α is degraded by hydroxylation of the HIF1 proline residues by proline hyrdoxylases (PHDs) through the von Hippel-Lindau (VHL)-mediated ubiquitination pathway. The inhibition of PHD promotes HIF1α to enter the nucleus and integrate with HIF1β to form heterodimers and promote the expression of HIF target genes such as glucose transporters (GLUTs) [119], and glycolytic enzymes [120, 121].

HIF1α is not only induced under hypoxic conditions, but has been shown to be up-regulated by NF-κB signaling in activated innate immune cells under normoxia [118, 122]. Moreover, T cells undergoing T_H17 differentiation have been found to express strong induction of HIF1α at the protein level, whereas the lowest level of expression was detected in Treg cells [5]. T cells deficient in HIF1α have an impairment in their

ability to induce T_H17 induction as shown by decreased levels of IL-17A, IL-17F, IL-21, and IL-22 and up-regulated expression of iTreg cells [5]. HIF1α deficient T cells have defects in IL-23R expression while T_H17 transcription factors RORγt and RORα are maintained [5]. Furthermore, deletion of HIF1α ameliorated the development of EAE in mice [5, 123]. These results demonstrate that HIF1α dependent activation of glycolysis functions as a key regulator in T_H17 differentiation.

T cell proliferation is also dependent upon the activity of (mTOR)-dependent pathways, which result in enhanced protein translation and aerobic glycolysis as well as the concomitant increase in the synthesis of biomolecules [124]. mTOR is an evolutionarily conserved serine and threonine kinase, which acts as an important upstream pathway to induce HIF1α transcription [5]. Rapamycin inhibits mTOR signaling via binding to mTOR complex 1, thereby reducing HIF-1α expression and IL-17, while favoring the induction of iTreg cells [5]. Rapamycin has also been found to ameliorate the clinical course of EAE [125-127]. Taken together, these studies highlight the critical role of the mTOR-HIF1α-glycolysis axis on T_H17 differentiation.

T cells also uptake essential amino acids from the environment to meet the metabolic demands of an immune response [128, 129]. Inhibition of amino acid metabolism in T cells results in anergic T cells with defective proliferation upon TCR stimulation [130]. Therefore amino acid abundance in the microenvironment and metabolism may also be key regulators for proper T cell mediated responses.

A recent study investigated a small molecule compound and the induction of the amino acid starvation response (AAR) [131]. The compound known as halofuginone (HF), which is derived from the plant alkaloid febrifugine, has been traditionally used in

Chinese medicine to reduce malarial fever [132]. Treatment of T cells with HF resulted in inhibition of T_H17 differentiation and promoted a cell fate switch to iTreg generation [131]. HF induces AAR by downregulating the expression of genes involved in amino acid transport, biogenesis, and protein synthesis, but the addition of excess amino acids can restore the defective T_H17 differentiation in HF treated cells [131]. Thus the regulation of amino acid availability also plays a critical role in T_H17 and iTreg differentiation decisions.

In addition, acetyl-CoA carboxylases (ACCs) have been found to play a critical role in iTreg and T_H17 differentiation. ACCs are key enzymes which regulate cellular fatty acid metabolism and exist as two isoforms in humans and other mammals as ACC1 and ACC2. Both enzymes are differentially expressed across tissues and catalyze the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA. ACC1 is found in the cytosol and crucial for *de novo* fatty acid synthesis, whereas ACC2 is associated with the outer mitochondrial membrane and regulates fatty acid oxidation [133]. Direct manipulation of ACC activity by soraphen A treatment (SorA), a specific inhibitor of both eukaryotic ACC isoenzymes [134, 135] under T_H17 inducing conditions resulted in a dose dependent reduction in the frequency of IL-17 producing cells, along with decreases in other T_H17 cell associated genes such as *IL17f, Stat3,* and *Hif1α* while enhancing iTreg induction [136]. Naïve cells differentiated under T_H17 inducing conditions with SorA treatment, did not however, downregulate the T_H17 specific transcription factor RORγt (encoded by *Rorc*) [136]. The reason for this may be due to the high level of up-regulation of Foxp3 expression which is known to antagonize RORγt function and thus T_H17 differentiation [87]. Therefore, direct manipulation of ACC

activity can be exploited due to the different metabolic processes between T effector cells like T_H17 cells and iTreg cells for immunomodulation of T cell responses.

The Warburg effect and the hexosamine pathway

In the quiescent state naïve T cells generate ATP from catabolic metabolism including oxidative phosphorylation of glucose, amino acids, and lipids in mitochondria. Contrarily, a defining feature of T cell activation is the switch from catabolism to anabolism that shifts energy production from the energy efficient oxidative phosphorylation to the less efficient aerobic glycolysis, as well as marked up-regulation in glutaminolysis, and biosynthetic activities, for rapid clonal expansion [128, 137-139]. The reliance on glycolysis even in the presence of high levels of oxygen, where glucose is fermented to lactate in the cytoplasm, generates a total of 2 ATP per glucose molecule. Aerobic glycolysis converts glucose into pyruvate, and then pyruvate reduction by lactate dehydrogenase (LDH) results in the production of lactate, which is excreted. This is far less efficient than oxidative phosphorylation, where glucose is metabolized to pyruvate, which then enters the tricarboxylic acid (TCA) cycle and the mitochondrial respiratory chain. This nets a total of 36 ATP per glucose molecule. The phenomenon of altered metabolism is known as the Warburg effect, and is a characteristic of cancer cells and rapidly proliferating cells such as T cells [1, 137, 140, 141]. The reason for this shift in energy production has long been attributed to facilitating the uptake and incorporation of nutrients into the biomass required to produce a daughter cell [2, 3]. However, nucleotide, lipid, and protein biosynthesis account for less than 10% of the enhanced glucose uptake [142, 143]. Furthermore,

cells such as DCs switch from oxidative phosphorylation to aerobic glycolysis upon Toll-like receptor (TLR) induced activation but do not proliferate [144]. Thus, the exact reasons and mechanism behind the Warburg effect remain elusive.

N-glycan biosynthesis requires glucose for *de novo* synthesis of UDP-GlcNAc, which begins with the conversion of fructose-6-phosphate to glucosamine-6-phosphate by Glutamine-Fructose-6-Phosphate Transaminase (GFAT) (**Fig. 1.2**.). However, fructose-6-phosphate is derived from glucose by the action of hexokinase/glucokinase (HK) followed by Phosphoglucose Isomerase (GPI) and is also the critical entry step into glycolysis. Thus, the hexosamine pathway and glycolysis may directly compete for fructose-6-phosphate (Figure 1a). However, the hexosamine pathway has not been investigated as a target of the Warburg effect.

Summary

It is well established that the galectin-glycoprotein lattice plays a critical role in T cell growth and differentiation. Metabolism, nutrient status, and environmental factors converge to influence N-glycan branching and regulate differentiation into T cell effector subsets. However, emerging evidence has begun to highlight the fundamental importance of metabolism in T cell differentiation. As the Warburg effect enhances glycolysis and increases consumption of fructose-6-phosphate, this may directly impact UDP-GlcNAc flux into the N-glycan branching pathway and thus impact differentiation into effector T cell subsets. The N-glycan branching pathway has not been investigated as a target of the Warburg effect, and is the major focus of this work.

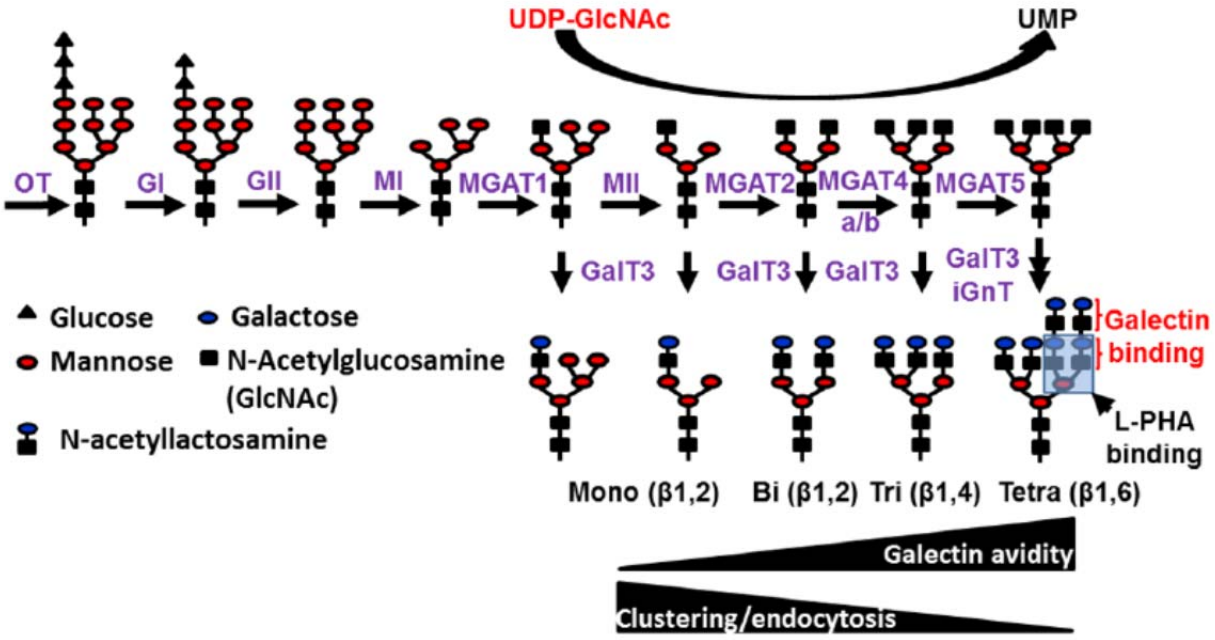

Figure 1.1 The N-glycosylation branching pathway. Oligosaccharyltransferase transfers a pre-assembled glycan, $Glc_3Man_9GlcNAc_2$, to the NxS/T motifs on glycoproteins in the ER. As glycoproteins transit through the Golgi, the N-acetylglucosaminyltransferase enzymes (Mgat1, Mgat2, Mgat4, and Mgat5) act in a sequential manner to generate branched N-glycans that display increasing affinities for galectins. Galactosyltransferase 3 extends the branches by adding a galactose to GlcNAc creating N-acetyllactosamine which can be bound by galectins. L-PHA binding sites are indicated. GI, glucosidase I; GII, glucosidase II; MI, mannosidase I; MII, mannosidase II; GalT3, Galactosyltransferase 3.

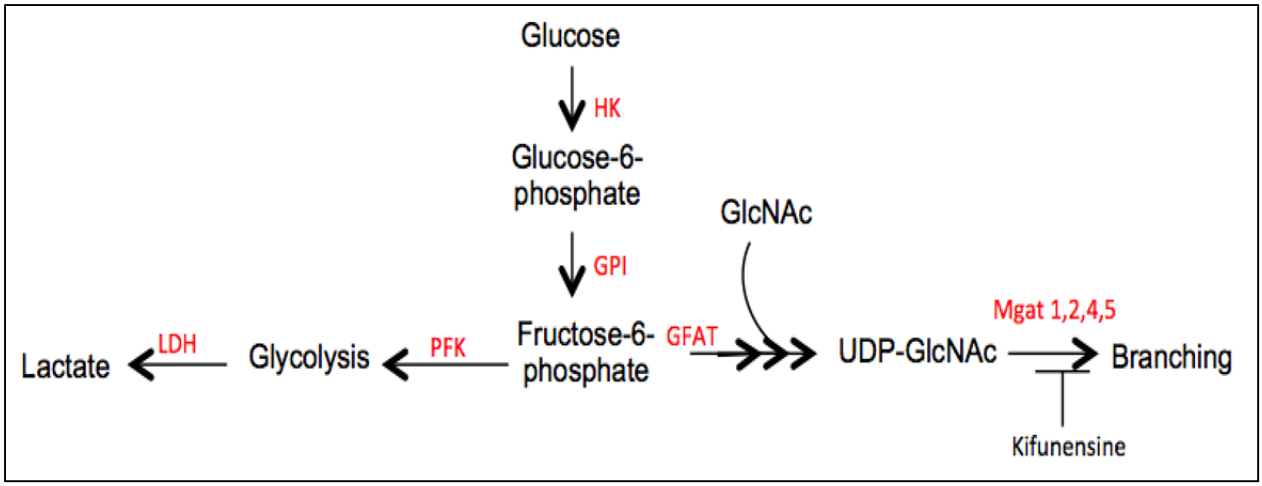

Figure 1.2 Glycolysis and the hexosamine pathway compete for fructose-6-phosphate. Fructose-6-phosphate is derived from glucose by the action of Hexokinase (HK) followed by Glucose-6-phosphate isomerase (GPI), in glycolysis. *De Novo* synthesis of UDP-GlcNAc begins with the conversion of fructose-6-phosphate to glucosamine-6-phosophate by Glutamine-Fructose-6-Phosphate Transaminase (GFAT) to then regulate N-glycan branching via (Mgat1, Mgat2, Mgat4, Mgat5). Glycolysis and the hexosamine pathway compete for fructose 6-phosphate via Phosphofructose kinase (PFK) and GFAT respectively. The ladder being further metabolized to lactate by lactate dehydrogenase (LDH).

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Chapter 2

The Warburg effect controls cell growth and differentiation by altering N-glycosylation

ABSTRACT

Rapidly proliferating cells such as T cells undergo the Warburg effect [1-3], a long-standing and poorly understood phenomenon that increases glucose uptake yet paradoxically reduces energy from glucose via a switch from oxidative phosphorylation to aerobic glycolysis. This has been rationalized as increased requirements for glucose-derived metabolites in biomass generation and/or limiting oxidative stress [4-7]. Asn (N)-linked protein glycosylation requires glucose and multiple glucose-derived sugars [8], yet surprisingly this pathway has not been investigated as a target of the Warburg effect. Here we report that the Warburg effect drives T cell growth and differentiation by controlling glucose flux to Golgi N-glycosylation. Branched N-glycans regulate the concentration and signaling of multiple surface receptors and transporters simultaneously to coordinate cellular and disease phenotypes [8-20]. Branching depends on UDP-GlcNAc biosynthesis via the hexosamine pathway [10, 11, 21, 22], a competitor of glycolysis for fructose-6-phosphate. In glycolytic T cells, the hexosamine pathway is starved of fructose-6-phosphate by glycolysis, limiting *de novo* UDP-GlcNAc biosynthesis and N-glycan branching dependent IL-2 receptor-α (CD25) surface retention. Restoring branching or UDP-GlcNAc levels via salvage of N-acetylglucosamine does not alter the glycolytic state yet inhibits T cell growth and switches cell fate from pro-inflammatory T_H17 to anti-inflammatory induced T regulatory (iTreg) cells by restoring CD25 surface expression. Forcing oxidative phosphorylation inhibits growth and drives iTreg over T_H17 differentiation by raising branching, demonstrating that glycolysis is not required for growth/differentiation control beyond its

regulation of branching. Thus, the Warburg effect primarily targets N-glycosylation via altered glucose flux to control T cell growth and differentiation.

INTRODUCTION

It has been over 90 years since Otto Warburg's seminal discovery that cancer cells display increased glucose uptake and a metabolic switch from oxidative phosphorylation to aerobic glycolysis [1, 2].The Warburg effect is now known to be a general characteristic of rapidly proliferating cells, yet its purpose remains poorly understood and controversial. The fermentation of glucose to lactate by aerobic glycolysis extracts 18 fold less energy compared to complete oxidation via the TCA cycle and the electron transport chain. Coupled with increased glucose uptake, this implies that altered glucose flux serves a critical purpose beyond energy production. Glucose metabolites produced by glycolysis prior to lactate are metabolic precursors for a number of macromolecular building blocks and may be used for biomass generation [4, 5]. However, nucleotide, protein and lipid synthesis account for <10% of the increase in glucose uptake [23, 24]. Alternatively, constraining oxidative phosphorylation in rapidly dividing cells may function to limit cellular damage from reactive oxygen species [6]. However, genetically blocking lactate production in lung cancer does not activate oxidative phosphorylation *in vivo* yet induces disease regression [7], questioning this hypothesis. Alternatively, the Warburg effect may have a direct regulatory function, as suggested in T cells for the promotion of IFNγ production by T cells and the differentiation into pro-inflammatory T helper 17 (T_H17) over anti-inflammatory T regulatory (Treg) cells [25-28]. However, these functions are cell type specific and fail to

define a global regulatory mechanism for the Warburg effect that is common to all rapidly proliferating cells.

Asn (N)-linked protein glycosylation is a feature of all metazoan cells. N-glycan biosynthesis requires glucose and multiple glucose-derived sugars [29], yet surprisingly this pathway has not been investigated as a target of the Warburg effect. In mammals, N-glycans play a critical role in regulating transitions between cell growth and arrest/differentiation [8-15]. N-acetylglucosamine (GlcNAc) branching of N-glycans couples with the number of N-glycans per protein molecule to regulate binding of glycoproteins to galectins, interactions that form a cell surface lattice that simultaneously controls the clustering, endocytosis, and signaling of multiple surface glycoproteins to effect cell function. Dysregulation of N-glycan branching has major impacts on diseases as diverse as immunity/autoimmunity [8, 12, 16-18, 30] cancer [19, 20, 31-34] and Type 2 Diabetes [35, 36]. N-glycan branching by the Golgi enzymes Mgat1, 2, 4, and 5 is regulated by biosynthesis of their shared substrate UDP-GlcNAc via the hexosamine pathway [10, 21]. *De novo* synthesis of UDP-GlcNAc begins with the conversion of fructose-6-phosphate to glucosamine-6-phosphate by Glutamine-Fructose-6-Phosphate Transaminase (GFAT), (**Fig. 1.1**). However, fructose-6–phosphate is derived from glucose by the action of hexokinase/glucokinase (HK) followed by Phosphoglucose Isomerase (GPI) and is also the critical entry step into glycolysis. Thus, the hexosamine pathway and glycolysis may directly compete for fructose-6-phosphate (**Fig. 1.1**). As the Warburg effect greatly increases consumption of fructose-6-phosphate by glycolysis, we hypothesized that aerobic glycolysis may starve

the hexosamine pathway of fructose-6-phosphate, lowering UDP-GlcNAc and N-glycan branching to control cell growth and/or differentiation.

RESULTS

N-glycan branching regulates T_H17 versus iTreg differentiation

To first examine our hypothesis, we investigated whether the Warburg effect regulates N-glycan branching during the differentiation of T cells into pro-autoimmune T_H17 cells. Induction of T_H17 differentiation induces the Warburg effect, but when glycolysis is inhibited, cell fate is switched from T_H17 to anti-autoimmune induced T regulatory cells (iTreg) cells despite T_H17 inducing cytokines [27]. If the Warburg effect starves the hexosamine pathway of fructose-6-phosphate, T_H17 cytokines are expected to reduce N-glycan branching via reduced biosynthesis of UDP-GlcNAc. To evaluate branching, we performed flow cytometry with L-PHA (*Phaseolus vulgaris, leukoagglutinin*), a plant lectin that binds specifically to β1,6 GlcNAc-branched N-glycans made by the Mgat5 enzyme and more generally, serves as a gauge of overall branching [8, 37] (**Fig. 1.1**). Strikingly, T_H17 inducing cytokines (TGFβ+IL-6+IL-23) acted synergistically to markedly reduce branching in activated T cells by ~ 3-4 fold, despite each cytokine individually having minimal effects (**Fig. 2.1a**). Although branching was reduced, mRNA expression of the Golgi branching enzymes Mgat1 and Mgat5 were increased (**Fig 2.1b,c**), consistent with reduced UDP-GlcNAc supply lowering branching. Indeed, while T cell activation markedly increases protein expression of GFAT and the critical glycolytic enzymes HK, GPI and Phosphofructose Kinase (PFK), GFAT is uniquely and specifically down-regulated by T_H17 cytokines

(**Fig. 2.2**). As GFAT and PFK directly compete for fructose-6-phosphate, the T_H17 cytokine induced reduction in GFAT should greatly favor glucose flux into glycolysis over the hexosamine pathway. Indeed, UDP-GlcNAc production is significantly reduced by T_H17 cytokines (**Fig. 2.3**). Moreover, directly restoring UDP-GlcNAc levels through the hexosamine salvage pathway by supplementing cells with the simple sugar GlcNAc (**Fig. 1.2**) [10, 21], thereby by-passing the requirement for fructose-6-phosphate, reverses the reduction in branching induced by T_H17 cytokines (**Fig. 2.4a**), markedly inhibits T_H17 differentiation and induces a cell fate switch to iTreg cells (**Fig. 2.4b**). The mannosidase I inhibitor kifunensine blocks branching (**Fig. 1.2**), (**Fig. 2.5a**) and reversed the effects of GlcNAc supplementation, confirming that raising UDP-GlcNAc levels blocked T_H17 and promoted iTreg differentiation by restoring N-glycan branching (**Fig. 2.5b**).

To verify this phenotype *in vivo*, 0.25 mg/ml GlcNAc was provided in the drinking water of mice after development of EAE induced by myelin oligodendrocyte glycoprotein (MOG) 35-55 peptide. GlcNAc was started on the second day of clinical symptoms and continued for the duration of the study [38]. This therapeutic approach significantly reduced clinical symptoms and increased N-glycan branching in T cells as seen by L-PHA staining (**Fig. 2.6, 2.7a**). Furthermore, compared with control mice, *in vivo* treatment with oral GlcNAc after disease onset also suppressed pro-inflammatory recall responses to the encephalitogenic MOG 35-55 peptide, with a significant reduction in secretion of IL-17 (**Fig. 2.7b**) as well as promotion of Treg cells (**Fig. 2.7c**).

To confirm this result genetically, we utilized the tet-on system to generate a mouse with inducible over-expression of the Golgi branching enzyme Mgat5

(ROSA^{rtTA}*Mgat5*^{tet}). Indeed, directly raising branching via transgenic over-expression of *Mgat5* also induced a cell fate switch from T_H17 to iTreg cells under T_H17 inducing conditions (**Fig. 2.8**). Blocking branching should have the opposite effect and indeed, inhibiting branching by targeted deficiency of the *Mgat1* (*Mgat1*$^{f/f}$/tetO-cre/ROSA-rtTA) [32] and *Mgat5* (*Mgat5*$^{-/-}$) [8] branching enzymes or with kifunensine significantly enhanced T_H17 induction (**Fig. 2.9a,b**), (**Fig. 2.5b**).

N-glycan branching determines T_H17 versus iTreg cell fate by regulating IL-2Rα

Interleukin-2 (IL-2) suppresses T_H17 and promotes iTreg differentiation [39, 40]; therefore we investigated whether branching promotes IL-2 signaling by enhancing surface retention of the IL-2 receptor. Blocking branching during T_H17 induction via *Mgat1* deletion markedly reduced surface expression of CD25 (**Fig. 2.10**), the alpha subunit of the IL-2 receptor. Up-regulation of branching via GlcNAc supplementation or *Mgat5* over-expression had the opposite effect, raising CD25 surface levels (**Fig. 2.11 a,b**). In contrast, IL-2 cytokine levels were unchanged by GlcNAc or kifunensine under T_H17 inducing conditions (**Fig. 2.12a**). The IL-2 receptor signals via STAT5 and this is markedly reduced by T_H17 cytokines (**Fig 2.12b**). GlcNAc supplementation restored pSTAT5 signaling despite T_H17 conditions (**Fig. 2.12b**), while sequestering IL-2 with anti-IL-2 antibody blocked the ability of GlcNAc to switch cell fate from T_H17 to iTreg (**Fig. 2.13a**). Similarly, lowering branching with kifunensine blocked the ability of exogenous IL-2 to induce a cell fate switch from T_H17 to iTreg (**Fig. 2.13b,c**). Taken together, these data demonstrate that UDP-GlcNAc and branching are decreased by

T_H17 cytokine induced down-regulation of GFAT, thereby lowering CD25 expression and IL-2 signaling to drive T_H17 over iTreg differentiation.

Glycolysis regulates N-glycosylation to control T_H17 versus iTreg cell fate

Next we investigated whether aerobic glycolysis was required for T_H17 differentiation. Remarkably, the cell fate switch from T_H17 to iTreg induced by GlcNAc supplementation did not alter the glycolytic state of the cells, as measured by the extracellular acidification rate and oxygen consumption rate (**Fig. 2.14**). As salvage of GlcNAc into the hexosamine pathway occurs downstream of fructose-6-phosphate, this result suggests that catabolism of fructose-6-phosphate to lactate is not necessary to drive T_H17 differentiation. To confirm this, we directly limited glucose flux into glycolysis by forcing oxidative phosphorylation during T_H17 differentiation using media with galactose [25] or the glycolysis inhibitor rapamycin. Rapamycin inhibits mTOR (mechanistic Target of Rapamycin) signaling via binding to mTOR complex 1, thereby reducing HIF-1α expression [41], a transcription factor that up-regulates glucose transporters and glycolysis enzymes including HK, PGM, and PFK [42]. Inhibiting glycolysis should promote fructose-6-phosphate entry into the hexosamine pathway over glycolysis to drive UDP-GlcNAc production, branching and iTreg differentiation despite T_H17 inducing conditions. Indeed, galactose and rapamycin enhanced branching (**Fig. 2.15a,b**). Moreover, the glycolytic inhibitors all drove iTreg over T_H17 differentiation; however their effects were reversed by the Golgi branching inhibitor kifunensine as well as *Mgat1* deficiency (**Fig. 2.16a-d**). This confirmed that the increase in branching induced by blocking glycolysis was necessary to inhibit T_H17 and promote

iTreg differentiation. Importantly, kifunensine did not alter the inhibition of aerobic glycolysis induced by galactose and rapamycin (**Fig. 2.17a,b**), demonstrating that T_H17 differentiation does not require aerobic glycolysis. Others have used 2-deoxy-D-glucose as an inhibitor of glycolysis; however this molecule is the same as 2-deoxy-D-mannose and is known to directly inhibit N-glycan biosynthesis via incorporation of 2-deoxy-D-mannose into the lipid-linked oligosaccharide precursor for N-glycosylation [43]. Indeed, 2-deoxy-D-glucose/2-deoxy-D-mannose markedly reduced N-glycan branching and promoted T_H17 differentiation (**Fig. 2.18a,b**) Taken together, these data demonstrate that a primary function of the Warburg effect during T_H17 differentiation is to lower N-glycan branching by starving the hexosamine pathway of fructose-6-phosphate substrate availability.

Branching regulates iTreg differentiation

In contrast to the multiple cytokines required for T_H17 differentiation, iTreg cells are physiologically induced by TGFβ alone. iTreg cells are non-glycolytic and utilize oxidative phosphorylation for ATP production [27, 28]. Our results with glycolytic T_H17 cells predict that the lack of aerobic glycolysis in iTreg cells should promote flux of fructose-6-phosphate into the hexosamine pathway to increase branching and iTreg differentiation via IL-2/CD25/STAT5 signaling. Indeed, reducing branching via *Mgat1* or *Mgat5* deficiency and/or with kifunensine all decreased iTreg differentiation induced by TGFβ alone (**Fig. 2.19a-c**), while raising branching with GlcNAc had the opposite effect (**Fig. 2.20**). Oligomycin is an inhibitor of oxidative phosphorylation that lowers cellular ATP. As PFK is allosterically inhibited by ATP, low doses of oligomycin should acutely

activate PFK and shift fructose-6-phosphate flux from the hexosamine pathway into glycolysis and thereby to inhibit branching and iTreg differentiation. Indeed, low doses of oligomycin (0.5mM) that have minimal impact on proliferation [6] increased glycolysis, lowered branching and reduced iTreg differentiation; the latter reversed by GlcNAc salvage into the hexosamine pathway (**Fig. 2.21a-c**). Higher doses of oligomycin lowered glycolysis and blocked proliferation. Coupled with the observation that under T_H17 inducing conditions GlcNAc induced iTreg differentiation without significantly altering aerobic glycolysis in the cell (**Fig. 2.14**), these data indicate that oxidative phosphorylation is not critical for iTreg differentiation beyond its regulation of branching. Thus, oxidative phosphorylation promotes iTreg differentiation by promoting branching via flux of fructose-6–phosphate to the hexosamine pathway over glycolysis.

Inhibition of glycolysis in non-polarized T cell blasts enhances branching and inhibits growth

In the absence of polarizing cytokines, TCR stimulation also induces the Warburg effect in T cell blasts, with increased glucose uptake and a switch to aerobic glycolysis. We previously observed increased UDP-GlcNAc production and branching in non-polarized T cell blasts relative to resting naïve T cells [16], an effect opposite to T_H17 cells. Increased branching serves to inhibit T cell growth by reducing T cell receptor clustering/signaling and promoting surface retention of the growth inhibitor CTLA-4 [8, 10, 13, 16-18, 30]. However, in contrast to T_H17 and resting T cells, GFAT is markedly up-regulated in neutral T cell blasts (**Fig. 2.2**). This should increase competition with PFK for fructose-6-phosphate to promote UDP-GlcNAc production and branching. As

such, inhibiting glycolysis in neutral T cell blasts should further shift fructose-6-phosphate to the hexosamine pathway to raise branching and inhibit T cell growth. Indeed, inhibiting glycolysis with galactose and rapamycin enhanced branching and inhibited T cell growth, effects reversed with the Golgi branching inhibitor kifunensine (**Fig. 2.22a-c**). These data indicate that the Warburg effect promotes T cell growth by limiting glucose flux to the hexosamine/branching pathway.

DISCUSSION

The regulation of metabolic pathways in proliferating and differentiating cells remains poorly understood. Our data support a model where the primary function of the Warburg effect is to control the flux of glucose into the hexosamine pathway and thereby regulate N-glycan branching; which in turn directs cell growth and differentiation. The Warburg effect has two distinct components, namely increased glucose uptake and aerobic glycolysis. The competition between PFK and GFAT for fructose-6-phosphate is essential for determining whether the Warburg effect lowers or raises branching. In T_H17 cells, GFAT is limiting and PFK dominates, restricting glucose flux into the hexosamine pathway and lowering branching along with CD25 surface expression to drive T_H17 differentiation. In T cells blasts, GFAT is upregulated, allowing Warburg induced increases in glucose uptake to raise *de novo* UDP-GlcNAc production and branching; however aerobic glycolysis still limits the extent of glucose flux into the hexosamine pathway to promote growth. Thus, the Warburg effect may raise or lower branching depending on the relative activities of GFAT versus PFK. Oxidative

phosphorylation skews the competition for fructose-6-phosphate towards GFAT and N-glycan branching, thereby promoting CD25 surface expression and iTreg differentiation.

Given the importance of the competition between GFAT and PFK, understanding how these two enzymes are differentially regulated is an important area to investigate in the future. For example, the cell fate decision between iTreg and T_H17 cell differentiation is dependent upon the pro-inflammatory cytokine IL-6. HIF1α, a key regulator of the expression of glycolytic enzymes in T cells has been found to be induced by IL-6 in a dose dependent manner [27]. This indicates a critical role for IL-6 signaling in HIF1α induction. As iTreg cells display a higher level of branching than T_H17 cells, IL-6 may also be a direct inhibitor of GFAT expression.

Other pro-inflammatory and/or anti-inflammatory cytokines may also differentially regulate GFAT versus PFK. *Mgat5* deficient $CD4^+$ T cells display an increase in T_H1 cells producing IFNγ and a reduction in T_H2 cells secreting IL-4 [45]. T_H1 and T_H2 cells have also been found to display increased glycolytic activity compared to iTreg cells and a strong up-regulation of glucose metabolism [27]. Based on our findings with lower N-glycan branching driving T_H17 induction, the differentiation of T_H1 cells may also be accompanied by a decrease in GFAT expression compared to T_H2 cells, where higher branching is favored.

Recently, it has been shown that T cells activated and cultured in galactose have a defect in IFN-γ production compared to cells activated and cultured in glucose [25]. The expression of IFN-γ mRNA and protein expression of the lineage transcription factor T-bet which defines this subset was not changed compared to T cells activated in glucose, suggesting a block in translation [25]. It was found that the glycolytic enzyme

glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was binding to the 3' untranslated regions (UTRs) of IFN-γ mRNA and limiting the translation of this cytokine in $CD4^+$ T cells cultured in galactose [25]. However, using a small interfering RNA (siRNA) approach to decrease GAPDH expression ~50% only resulted in a <10% increase in IFN-γ compared to cells expressing scrambled siRNA [25]. The authors attributed this to only a modest siRNA mediated decrease in gene expression due to the abundance of this transcript [25]. Based on our model, we predict that activated $CD4^+$ T cells differentiated under T_H1 inducing conditions cultured in galactose will display enhanced fructose-6-phosphate flux towards the hexosamine pathway, thus raising N-glycan branching and inhibiting T_H1 differentiation and IFN-γproduction; effects expected to be reversed by kifunensine or *Mgat1* deficiency. Under glycolytic conditions, where GAPDH is not bound to the 3' UTR of IFN-γ, siRNA targeting of GAPDH is also expected to reduce flux of fructose-6-phosphate into glycolysis and thereby promote branching and inhibit IFN-γ mRNA expression. siRNA knockdown of GFAT should have the opposite effects.

The ability of GlcNAc to by-pass the Warburg effect in glycolytic T_H17 cells and promote iTreg cell differentiation further validates its therapeutic potential as a treatment of pro-inflammatory diseases such as Multiple Sclerosis. iTreg cells are non-glycolytic, however, differentiation of T_H17 cells in the presence of GlcNAc results in a cell fate switch to iTreg under glycolysis favoring conditions. In mice that have developed clinical myelin oligodendrocyte glycoprotein (MOG) 35-55-induced EAE, oral GlcNAc treatment on the second day of clinical symptoms significantly reduced clinical symptoms and IL-17. GlcNAc enters the cell through macropinocytosis and is exclusively salvaged into

the hexosamine pathway for production of UDP-GlcNAc [46]. Uptake of GlcNAc via macropinocytosis is enhanced in T cell blasts. Thus cells undergoing the Warburg effect may be more sensitive than other cells to this treatment, providing a degree of specificity. Our results identify the hexosamine pathway and metabolic flux as a critical regulator of differentiation and cell growth and these pathways should be further explored as a strategy for metabolic immune modulation. Indeed, GlcNAc may be useful in any inflammatory disease where a cell fate switch from T_H17 to iTreg would be therapeutic.

MATERIALS and METHODS

Mice.

Mgat1$^{f/f}$ (*lox*P-flanked *Mgat1* gene, #006891), tetO-cre (tetracycline-responsive promoter element driving Cre recombinase expression, #006234) and ROSA26-rtTA (reverse tetracycline trans-activator expression driven by the ROSA26 promoter, #006965) mice were obtained from Jackson Laboratories. All of these mice were on the C57BL/6 background and interbred to generate all other mice. To delete Mgat1 in peripheral T cells, doxycycline in 1% sucrose was provided in the drinking water at 2 mg/ml to *Mgat1*$^{f/f}$tetO-Cre^{+}ROSA26-rtTA mice. The transgene expression vector pTRE-Tight (clontech) which contains a tet regulated promoter controlling the expression of subcloned Mgat5 cDNA (a gift from Jim Dennis) was used to generate the tetO-*Mgat5* line. The transgene construct was cut from the transgene expression vector and DNA microinjected into fertilized mouse eggs. Founder mice were bred with mice of the same strain and transgenic offspring were routinely identified by PCR using a primer pair

derived from the Tet promoter and Mgat5 transgene. Transgenic positive mice were then crossed with ROSA26-rtTA to generate tetO-*Mgat5*/rtTA^{+} mice. To overexpress *Mgat5* in mouse splenic CD4^{+} T cells, doxycycline was added to cell culture at (0.5-4μg/mL^{-1}).

Mouse T cell cultures.

Naïve CD4^{+} T cells were isolated from spleens and enriched by EasySep Mouse CD4^{+} T cell isolation kit and used in all experiments (StemCell Technologies). For T_H17 cell induction cells were cultured for 4 d with plate-bound anti-CD3ε (0.25-5μg mL^{-1}, clone 145-2C11; eBioscience), anti-CD28 (2μg mL^{-1}, clone 37.51; eBioscience), anti-IFNγ (10μg mL^{-1}, clone XMG1.2; eBioscience), anti IL-4 (10μg mL $^{-1}$, clone 11B11; eBioscience), rhTGF-β1 (5ng mL $^{-1}$; eBioscience), rmIL-6 (20 ng mL^{-1}; eBioscience), and rmIL-23 (20ng mL^{-1}; eBioscience). For Treg cell induction naïve T cells were cultured for 4 d in the presence of plate bound anti-CD3ε (1μg mL^{-1}), anti- CD28 (2μg mL^{-1}), and rhTGF-β1 (0.625-10ng mL^{-1}). For pharmacological inhibitor treatments cells were incubated with vehicle, 1nM Rapamycin (EMD Milipore), and (0.5nM) Oligomycin-A (Sigma). For experiments using GlcNAc treatment was 40mM added to culture media daily. Cytokine production in cell culture supernatants was analyzed by enzyme-linked immunosorbent assay (ELISA) with mouse Ready Set Go! IL-2 assay kits (ebioscience) according to the manufacturer's instruction.

Western blot.

For all expression, cells were stimulated by plate bound anti-CD3ε plus anti-CD28 for 72 h before lysis or left unstimulated. Unstimulated cells were cultured for 12 h in medium prior to cell lysis. Whole-Cell lysates were prepared and supplemented with Halt Protease Phosphotase Inhibitor (Fisher). Cell lysates were separated by SDS-gel electrophoresis and transferred to nitrocellulose membranes (Life Technologies). Antibodies to Hexokinase I(C3C54) (#2024), Phospho-Stat5 (D47E7) (#4322), PFKL (#8175), actin (#4970), Hsp90 (#4877), and HRP-conjugated rabbit IgG (#7074) were from Cell Signaling Technology. Antibodies to PFK-P (PA5-28673) and GPI (PA5-26787) were from Life Technologies. PFK-M (#842735) was from R&D systems. GFAT (#EPR4854) was from LifeSpan Biosciences. Hexokinase I, PFKP, PFKL, PFKM, anti-GFPT1, anti-GPI abundance were calculated by normalization to actin or Hsp90 and relative to control stimulated, or unstimulated cells as described.

Metabolism analysis.

Cells were harvested after 48 h of culture and plated on 24-well XF cell culture micro-plates coated with Cell-Tak (Corning) at a concentration of 22.4μg mL^{-1} in nonbuffered RPMI 1640 (containing either 10mM glucose or galactose, 2mM L-glutamine, and 1mM sodium pyruvate) (Sigma) at a density of 7.5 x 10^5 cells per well. Using two step plating, microplates were incubated for 10 min with 100μl of cells in nonbuffered media at 37C in a non-CO_2 incubator. Then 400μl of nonbuffered media was added to each well and incubated for an additional 20 min. The extracellular acidification rate (ECAR) and Oxygen Consumption Rate (OCR) was measured using an XF24 Extracellular Flux

Analyzer (Seahorse Biosciences) under basal conditions and in response to mitochondrial inhibitors using subsequent injections at a final concentration of glucose (10mM), oligomycin (1μM) and 2-deoxyglucose (2-DG; 100mM).

Flow cytometry and intracellular staining.

Monoclonal antibodies specific to the following mouse antigens (and labeled with the indicated fluorescent markers) were purchased from eBioscience: IL-17 PE (eBio17B7), Foxp3 APC (Fjk-16s), CD4 PrpCy5.5 (RM4-5), CD69 PE (H1.2F3), CD25 PE (PC61.5), 7-AAD. In addition, FITC L-PHA (vector labs) was used to stain cells. For intracellular staining cells were stimulated at 37C for 5 h with phorbol 12-myristate 13-acetate (50ng mL^{-1}; Sigma-Aldrich) and ionomycin (750 ng mL^{-1} Sigma-Aldrich) in the presence of the GolgiPlug (1μl per 1mL cell culture) (BD Biosciences) and stained using Foxp3/ Transcription Factor Fixation/Permeabilization Kit (eBioscience) according to the manufacturer's instruction. Flow cytometry experiments were performed with BD FACS Calibur, LSR II, Attune Acoustic Focusing Cytometry.

UDP-GlcNAc measurement by LC-MS/MS.

Isolated splenic $CD4^+$ T cells were activated in culture in the presence or absence of T_H17 inducing conditions as described above. After 72 hours in culture, cells were harvested, washed twice with ice-cold 1x PBS and counted. 15^6 cells were pelleted in 1.5 ml Eppendorf tubes by centrifuging at 350g for 7 minutes at 4 °C. Metabolites were extracted from the pellets by the addition of 1 mL of ice-cold solution of 40% acetonitrile, 40% methanol, and 20% water. The tubes were vortexed for 1 h at 4 °C and spun down

at 14000 rpm for 10 min at 4 °C (Eppendorf, Germany). The supernatant was transferred to fresh tubes and evaporated to dryness in an Eppendorf Vacufuge at 30 °C (Eppendorf, Germany). The dry samples were stored at -80 °C until analysis at which point they were reconstituted in 100µL of LC-MS grade water. The samples were run on a Waters (Micromass) Quattro Premier XE LC-MS/MS machine in negative mode with Water:Acetonitrile (+ 0.2% Acetic Acid and 5mM Ammonium Acetate) solvent. UPLC with C18 reverse phase column was employed. Area was converted to UDP-GlcNAc concentration by plotting on a purified UDP-GlcNAc (Sigma) standard curve run simultaneously

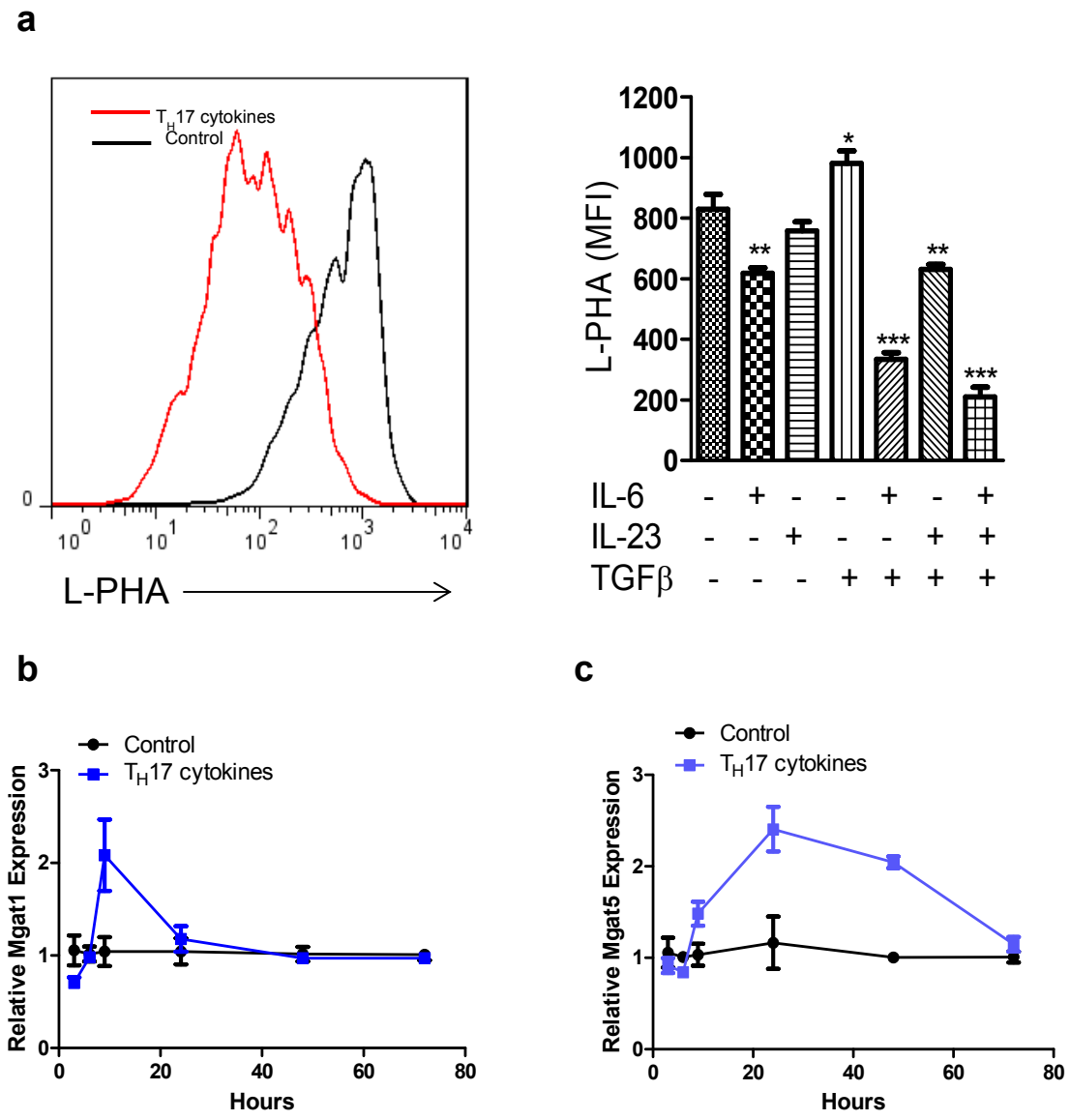

Figure 2.1 T_H17 cytokines synergistically downregulate N-glycan branching. (a) Extracellular L-PHA was measured on purified mouse splenic $CD4^+$ T-cells activated with anti-CD3/28 for 4 days and treated with anti-IFN-γ (10 μg mL^{-1}) and anti-IL-4 (10 μg mL^{-1}) along with the cytokines IL-6 (20ng mL^{-1}), IL-23 (20 ng mL^{-1}) and TGFβ (5 ng mL^{-1}).RT-PCR levels of Mgat1 (b) or Mgat5 (c) under Th17 inducing conditions as described in (a) harvested at 3, 6, 9, 24, 48, and 72h time points.

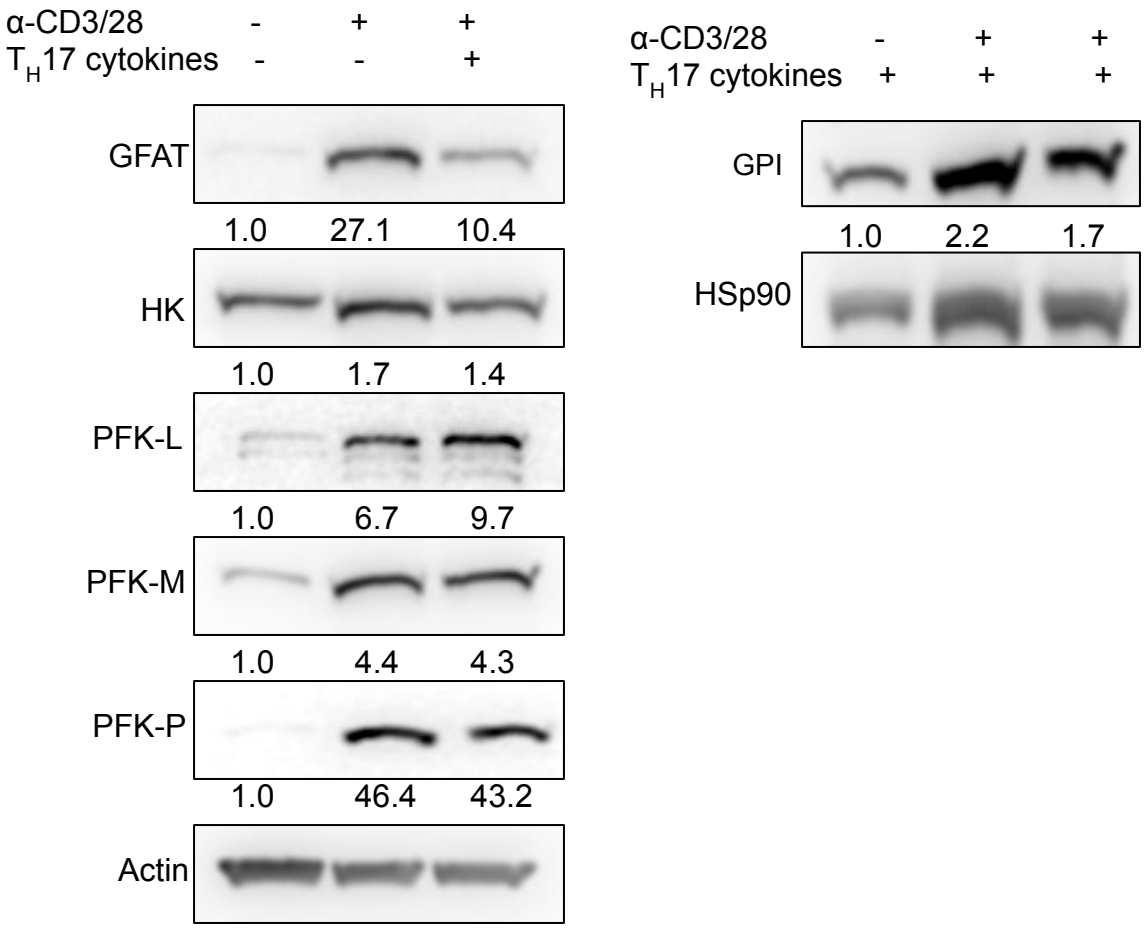

Figure 2.2 Western blot of the glycolytic enzymes and GFAT. Mouse splenic $CD4^+$ T-cells activated with anti-CD3/28 for 3 days and treated with anti-IFN-γ (10 μg mL^{-1}) and anti-IL-4 (10 μg mL^{-1}) with and without the cytokines IL-6 (20ng mL^{-1}), IL-23 (20 ng mL^{-1}) and TGFβ (5 ng mL^{-1}). Unstimulated mouse splenic $CD4^+$ T cells were isolated and plated for 12h before harvested and lysed using RIPA buffer. Western blot of: GFAT, HK, GPI, PFK-L, PFK-M, PFK-P.

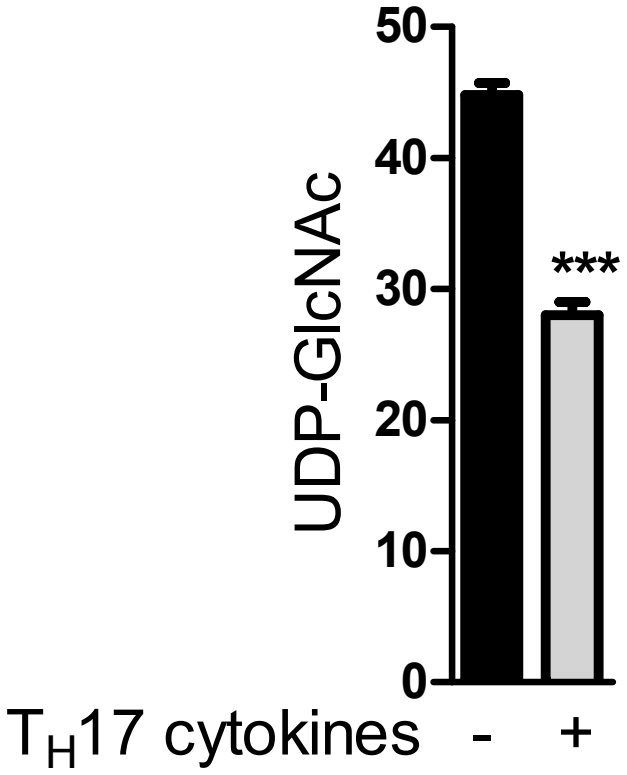

Figure 2.3 LC-MS/MS of UDP-GlcNAc levels. Mouse splenic $CD4^+$ T-cells activated with anti-CD3/28 for 3 days and treated with anti-IFN-γ (10 μg mL^{-1}) and anti-IL-4 (10 μg mL^{-1}) with and without the cytokines IL-6 (20ng mL^{-1}), IL-23 (20 ng mL^{-1}) and TGFβ (5 ng mL^{-1}). Metabolites were extracted and UDP-GlcNAc was quantified by LC-MS/MS.

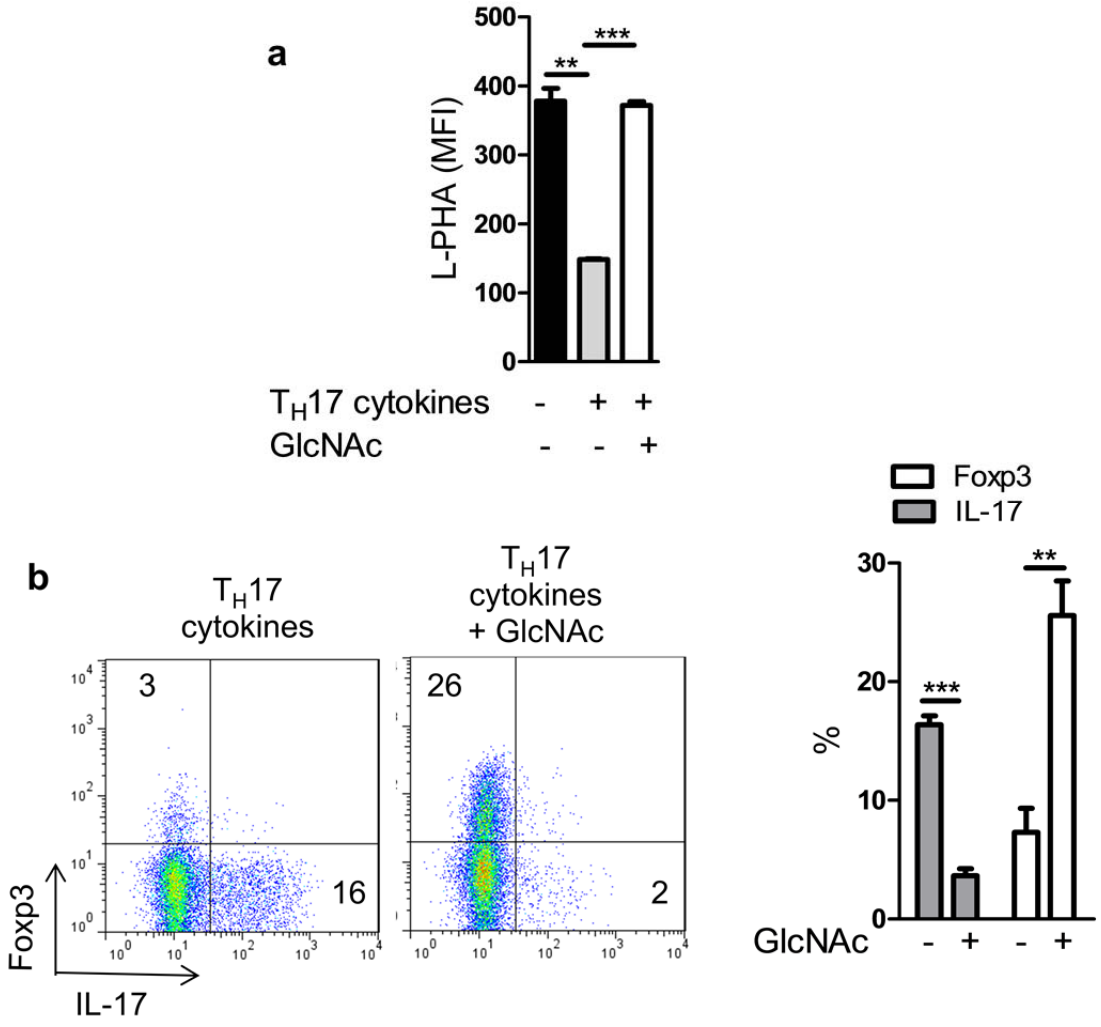

Figure 2.4 GlcNAc supplementation raises branching and induces a cell fate switch. (a) Flow cytometric ananlysis of extracellular L-PHA was measured on purified CD4$^+$ T cells with and without T_H17 inducing conditions and 40mM GlcNAc treatment added daily to culture media and harvested at day 4. (b) Flow cytometric analysis of intracellular IL-17 and Foxp3 were measured in CD4$^+$ T cells activated with anti-CD3/28 for 4 days under T_H17 inducing conditions and treated with 40mM GlcNAc supplemented daily in culture media.

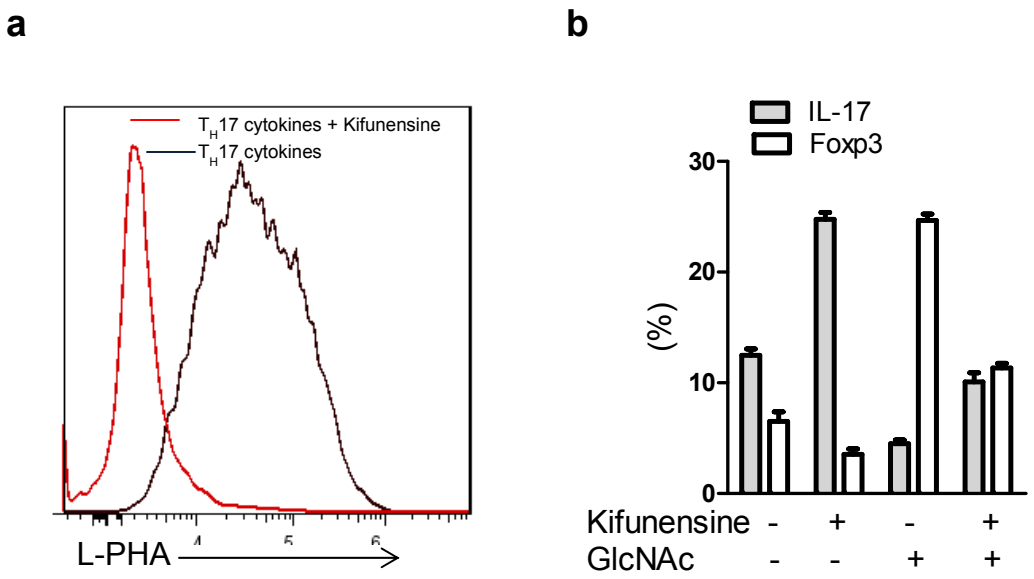

Figure 2.5 Kifunensine inhibits branching and reverses the effects of GlcNAc. (a) Flow cytometric ananlysis of extracellular L-PHA was measured on purified $CD4^+$ T cells with and without T_H17 inducing conditions and 10μM kifunensine treatment added at the time of plating to culture media and harvested at day 4. (b) Flow cytometric analysis of intracellular IL-17 and Foxp3 were measured in $CD4^+$ T cells activated with anti-CD3/28 for 4 days under T_H17 inducing conditions and treated with and without 40mM GlcNAc supplemented daily in culture media and with and without 10μM kifunensine added to culture media at the time of plating.

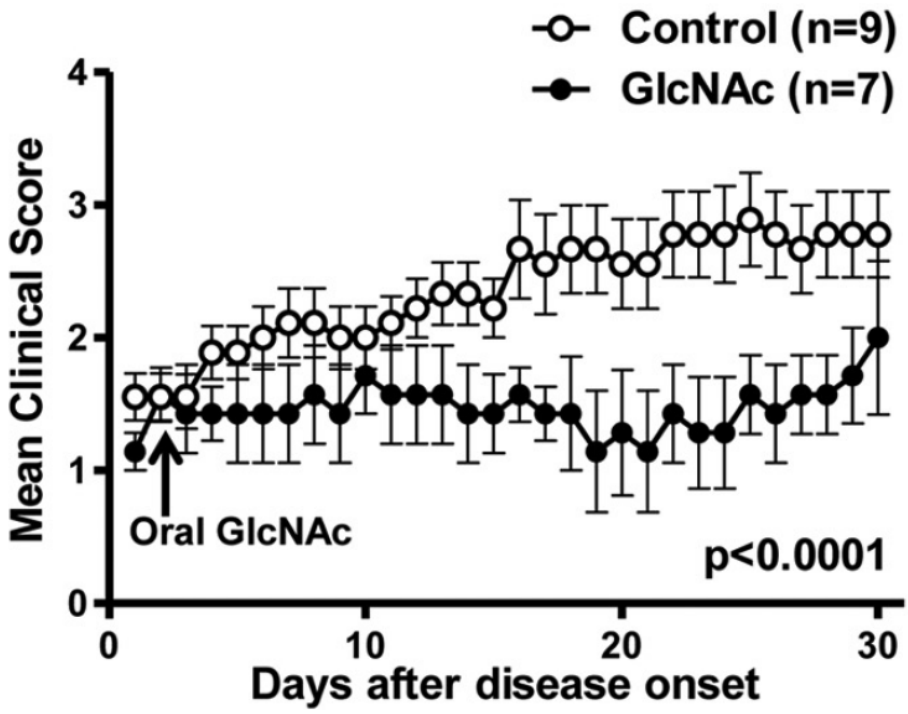

Figure 2.6 Oral GlcNAc treatment attenuates the clinical course of EAE. EAE was induced in C57BL/6 mice by immunization with MOG 35-55 peptide emulsified in complete Freund's adjuvant and pertussis toxin. Mice were treated orally with GlcNAc by supplementing the drinking water at 0.25 mg mL^{-1} starting on the second day after disease onset and continued for the duration of the study. (n = 9 per control group, n = 7 per GlcNAc group). Day 1 indicates the first day of disease onset. Mice were examined daily for clinical signs of EAE over the next 30 days with the observer blinded to treatment conditions and scored daily as follows: 0, no disease; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb weakness or paralysis and hindlimb paralysis; 5, moribund or dead. Mean clinical scores per group daily were compared by the Mann-Whitney *U* test.

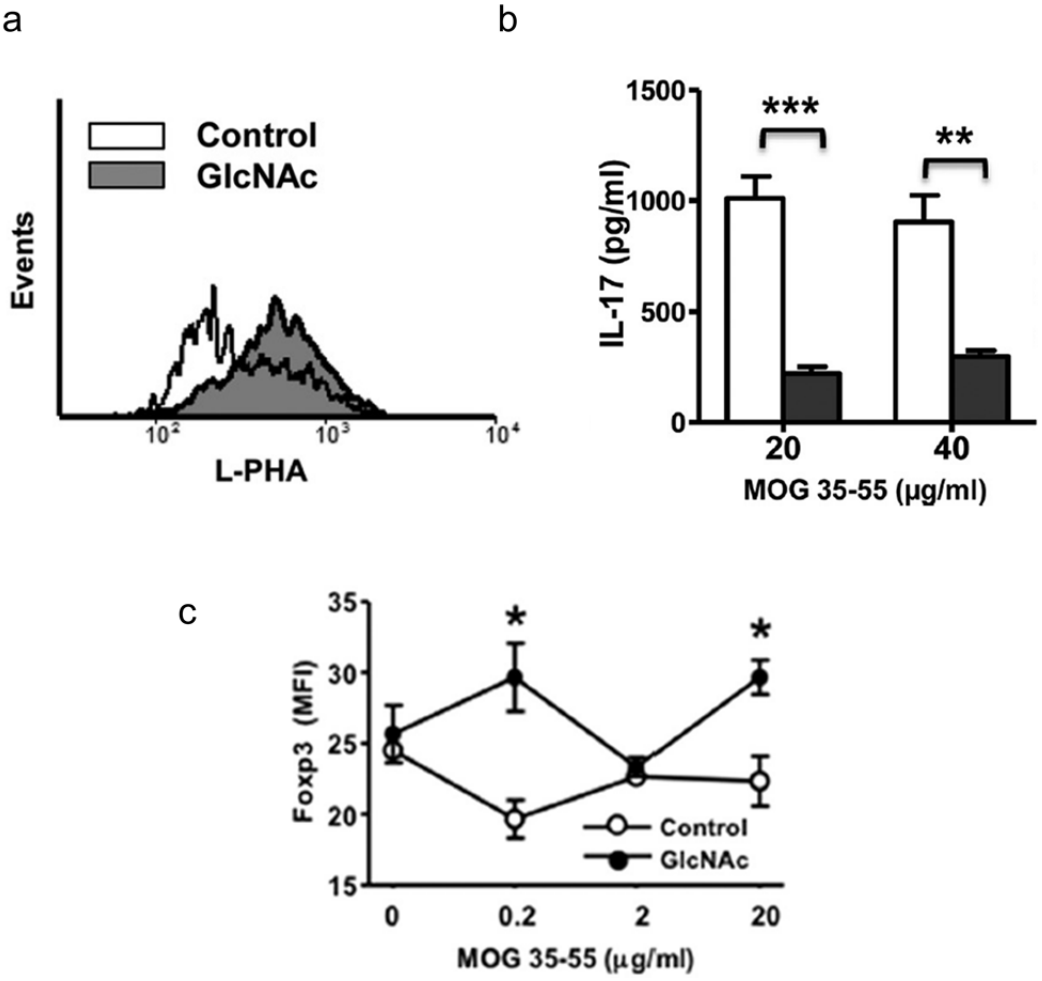

Figure 2.7 Oral GlcNAc treatment raises branching, reduces IL-17 secretion, and enhances Foxp3. (a) GlcNAc treatment increased N-glycan branching in T cells as seen by L-PHA staining in representative mice taken at the peak of disease. The results are representative of at least three mice compared from each group. (b-c) *in vivo* treatment with GlcNAc inhibited production of proinflammatory cytokine IL-17 upon restimulation with MOG 35-55 peptide *in vitro* (c) and enhanced Foxp3 expression.

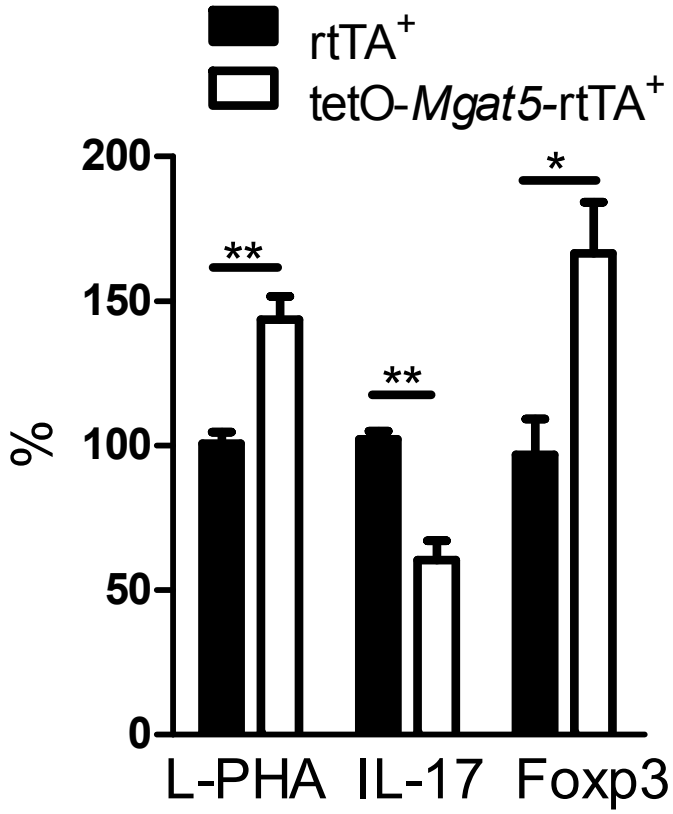

Figure 2.8 Over expression of *Mgat5* drives branching and induces a cell fate switch to iTreg. Flow cytometric analysis of rtTA$^+$ and tetO-*Mgat5*/rtTA$^+$ isolated splenic CD4$^+$ T cells activated with anti-CD3/28 under T_H17 inducing conditions and treated with 4µg mL^{-1} doxycycline at the time of plating for 4 days followed by surface staining of L-PHA and CD4 and intracellular staining of IL-17 and Foxp3.

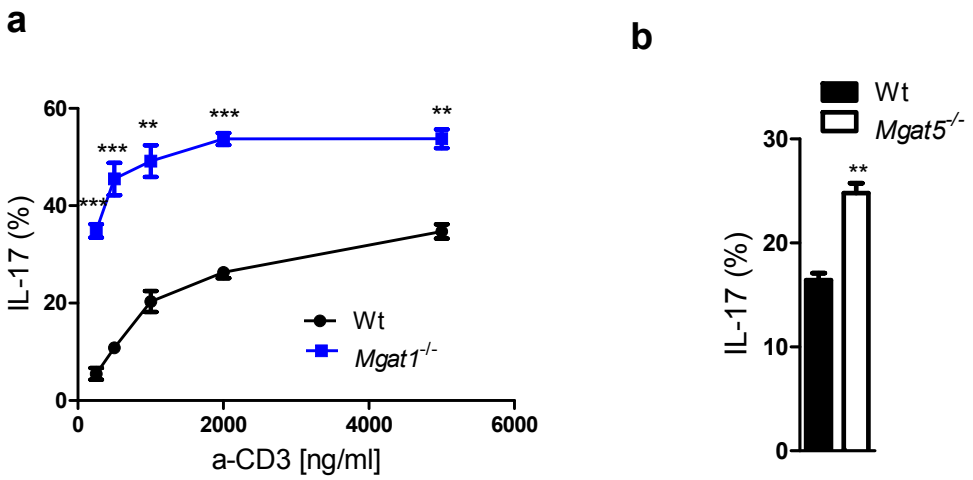

Figure 2.9 Deficiency of *Mgat1* and *Mgat5* enhance T_H17 induction. (a) Flow cytometric analysis of Wt and *Mgat1*$^{-/-}$ isolated splenic CD4^{+} T cells activated with anti-CD28 and anti-CD3 (250-5000ng mL^{-1}) under T_H17 inducing conditions for 4 days followed by intracellular staining of IL-17. (b) Flow cytometric analysis of Wt and *Mgat5*$^{-/-}$ isolated splenic CD4^{+} T cells activated with anti-CD3/28 under Th17 inducing conditions for 4 days followed by intracellular staining of IL-17.

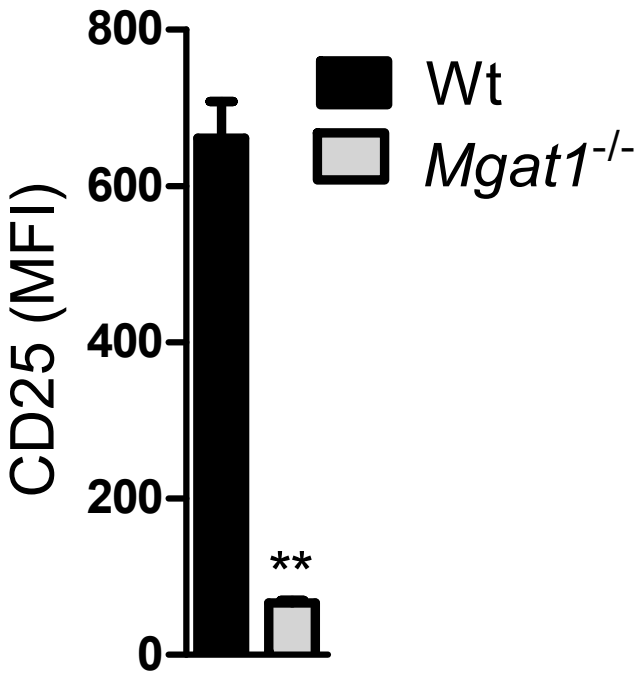

Figure 2.10 *Mgat1* deletion reduces CD25 surface expression. Flow cytometric analysis of Wt and *Mgat1*$^{-/-}$ isolated splenic CD4^{+} T cells activated with anti-CD3/28 under T_H17 inducing conditions for 4 days followed by extracellular staining of CD25. Data shown is gated on L-PHA low cells in the *Mgat1*$^{-/-}$ condition.

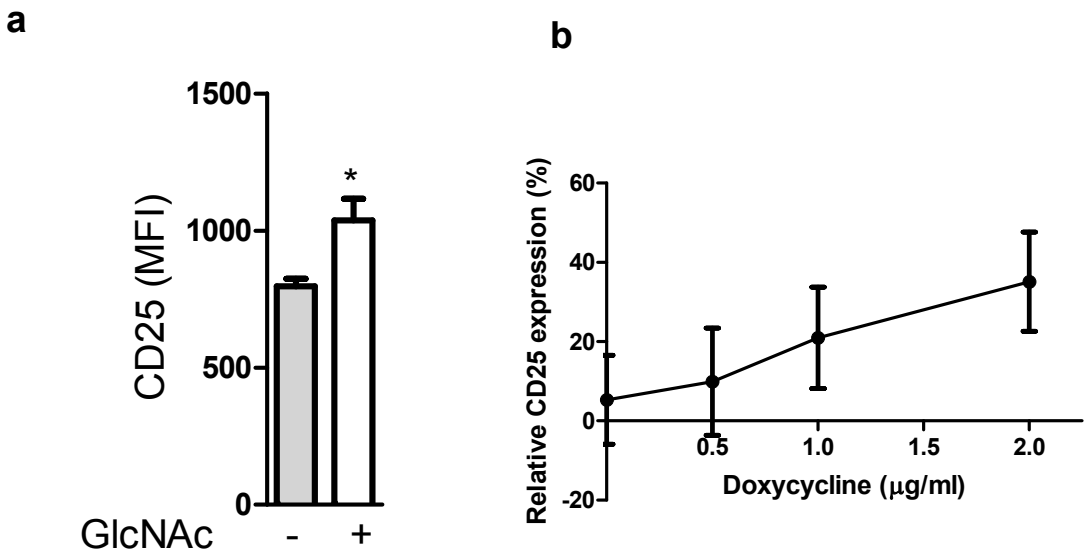

Figure 2.11 Raising branching enhances CD25 surface expression. (a) Flow cytometric analysis of extracellular CD25 measured on isolated $CD4^+$ $IL\text{-}17^+$ T cells treated with and without 40mM GlcNAc added daily to culture media for 4 days with anti-CD3/28 under T_H17 inducing conditions. (b) Flow cytometric analysis of extracellular CD25 measured on purified mouse splenic $CD4^+$ T cells from tetO-*Mgat5*-$rtTA^+$ and $rtTA^+$ mice activated with anti-CD3/28 for 4 days under T_H17 inducing conditions and treated with (0.5-2μg/mL) doxycycline at the time of plating .

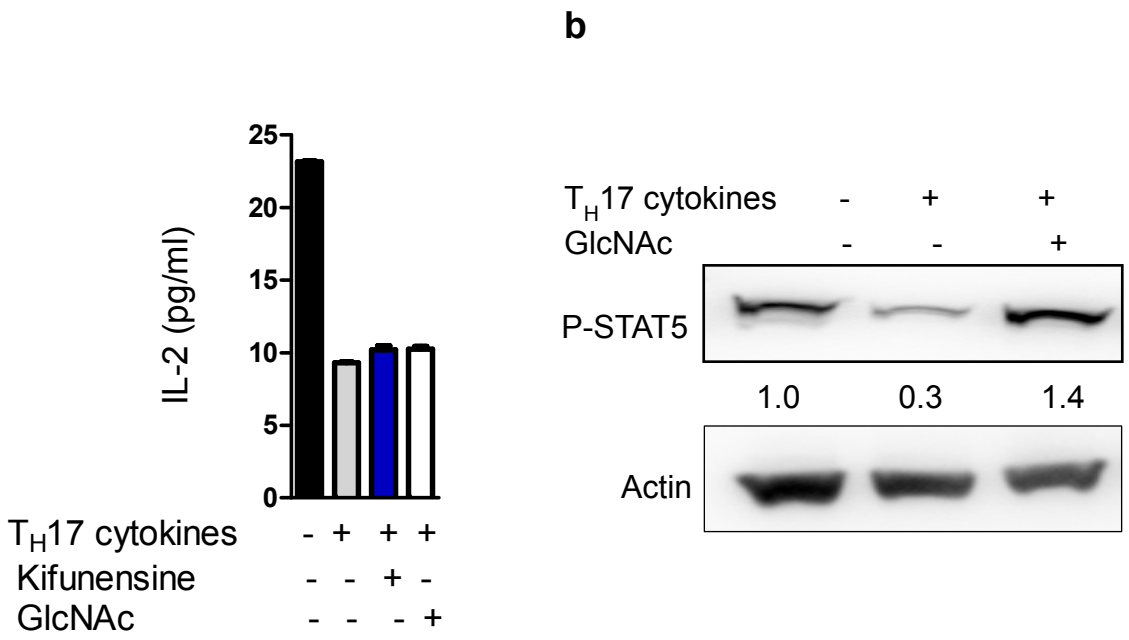

Figure 2.12 Raising branching does not alter IL-2 cytokine levels and enhances P-STAT5. (a) ELISA assay of IL-2 supernatant. Purified mouse splenic $CD4^+$ T cells were activated with anti-CD3/28 for 4 days with and without T_H17 inducing conditions and treated with and without 40mM GlcNAc supplemented daily in culture medium, or 10μM Kifunensine added at the time of plating. (b) Western blot analysis of P-STAT5 expression. Isolated splenic $CD4^+$ T cells activated with anti-CD3/28 for 3 days under T_H17 inducing conditions with and without 40mM GlcNAc treatment supplemented into culture media daily.

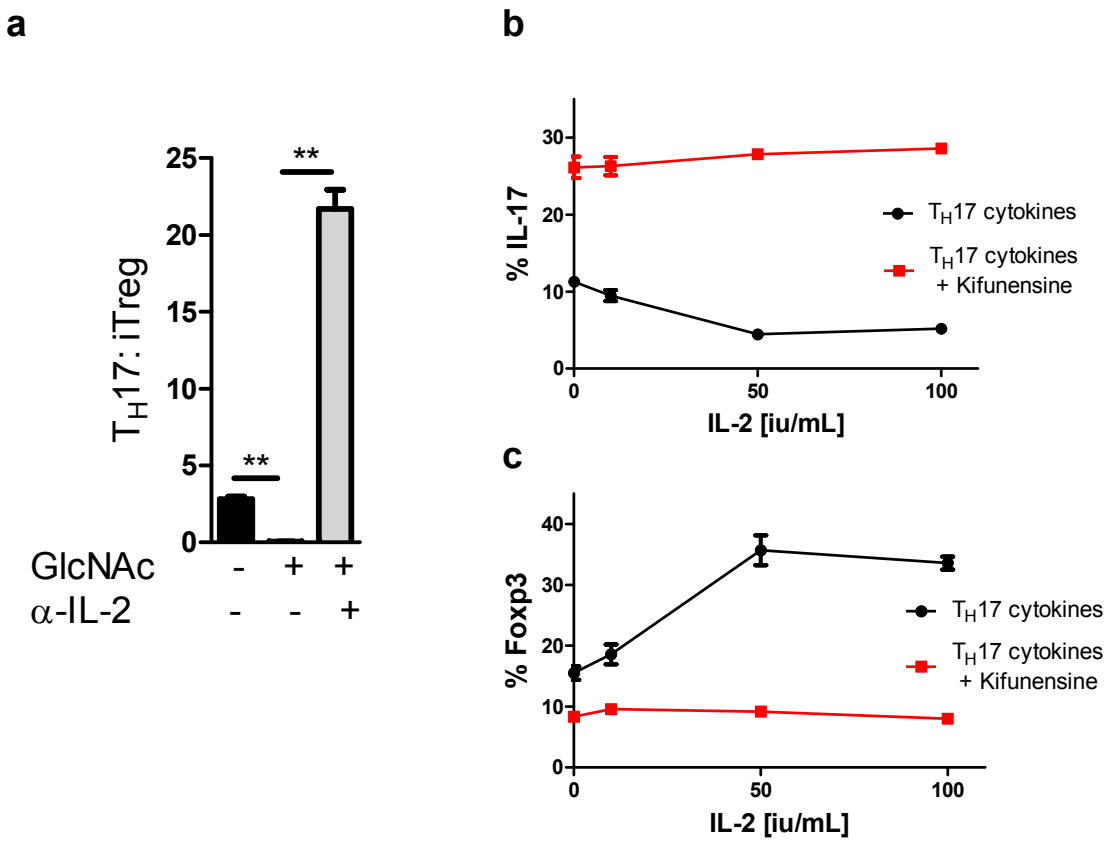

Figure 2.13 Sequestering IL-2 in the presence of GlcNAc or lowering branching prevents the cell fate switch from T_H17 to iTreg. (a) Flow cytometric analysis of isolated mouse splenic $CD4^+$ T cells were activated with anti-CD3/28 for 4 days under T_H17 inducing conditions and treated with or without anti-IL-2 (20μg mL^{-1}) or with and without 40mM GlcNAc supplementation to culture media daily followed by intracellular staining of IL-17 and Foxp3. (b) Flow cytometric analysis of Intracellular IL-17 and (c) Foxp3 measured in isolated $CD4^+$ T cells activated with anti-CD3/28 for 4 days under T_H17 inducing conditions and treated with (25-100 iU mL^{-1}) IL-2 with and without 10μM kifunensine.

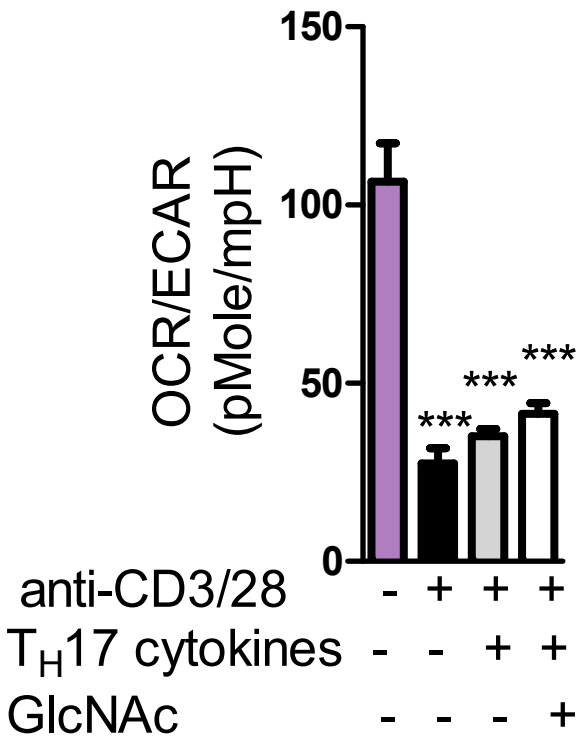

Figure 2.14 GlcNAc supplementation does not alter glycolysis. OCR and ECAR were measured by Seahorse in isolated splenic $CD4^+$ T cells harvested at 12 hours (unstimulated cells) and placed in culture media and cells activated with and without anti-CD3/28 for 48 hrs, with and without T_H17 inducing conditions and 40mM GlcNAc treatment.

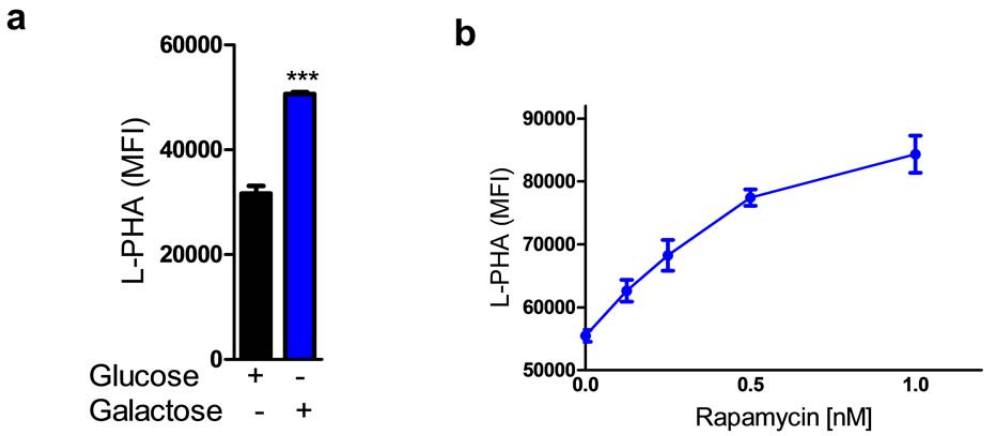

Figure 2.15 Inhibition of glycolysis enhances branching. (a) Flow cytometric analysis of extracellular L-PHA measured on splenic isolated $CD4^+$ T cells activated with anti-CD3/28 in media containing 10mM glucose (Glc) or 10 mM galactose (Gal) for 4 days under T_H17 inducing conditions, or (b) in glucose media treated with or without rapamycin (0-1nM).

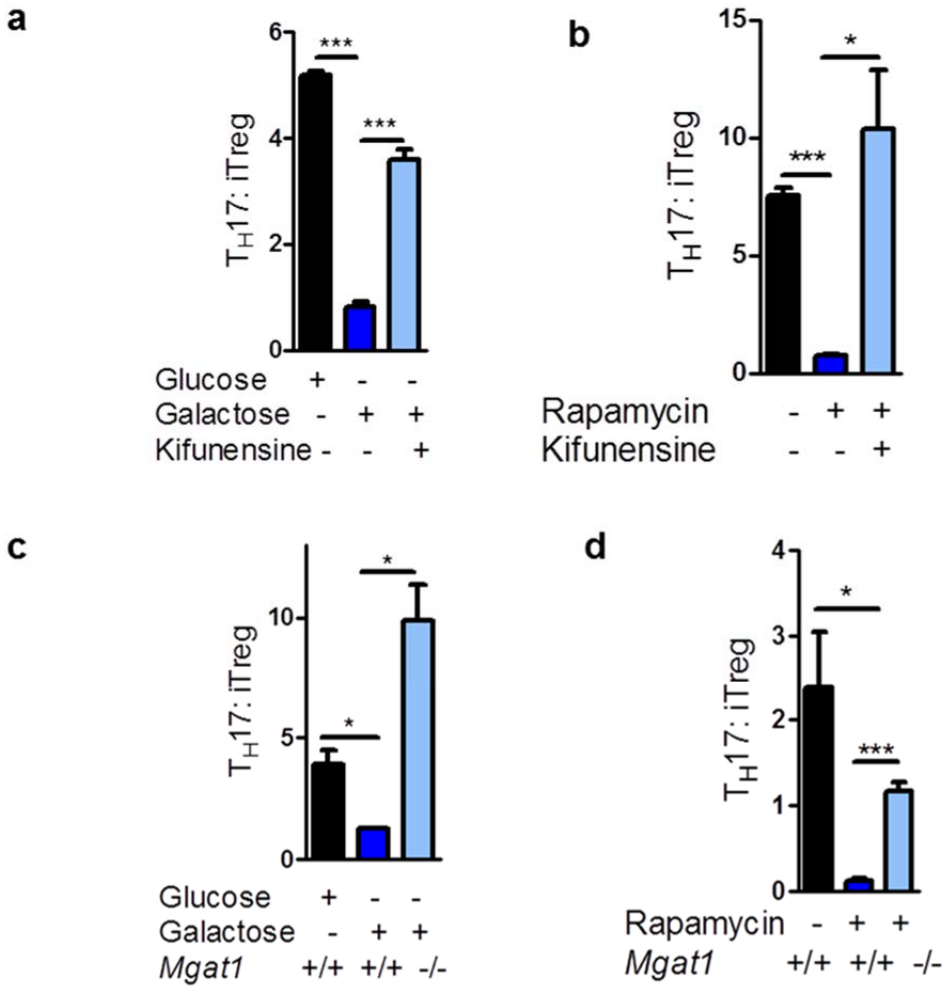

Figure 2.16 Inhibition of glycolysis induces a cell fate switch to iTreg which is reversed by branching deficiency (a-d) Flow cytometric analysis of Intracellular IL-17 and Foxp3 in isolated splenic $CD4^+$ T cells activated with anti-CD3/28 for 4 days under T_H17 inducing conditions in (a) Glc or Gal media and treated with 10μM kifunensine added to Gal media at the time of plating (b) treated with or without 1nM rapamycin or with and without 10μM kifunensine at the time of plating. (c-d) Intracellular IL-17 and Foxp3 in Wt and *Mgat1*$^{-/-}$ $CD4^+$ T cells, activated with anti-CD3/28 under T_H17 inducing conditions in (c) Glc or Gal media for 4 days, or (d) treated with and without 1nM rapamycin.

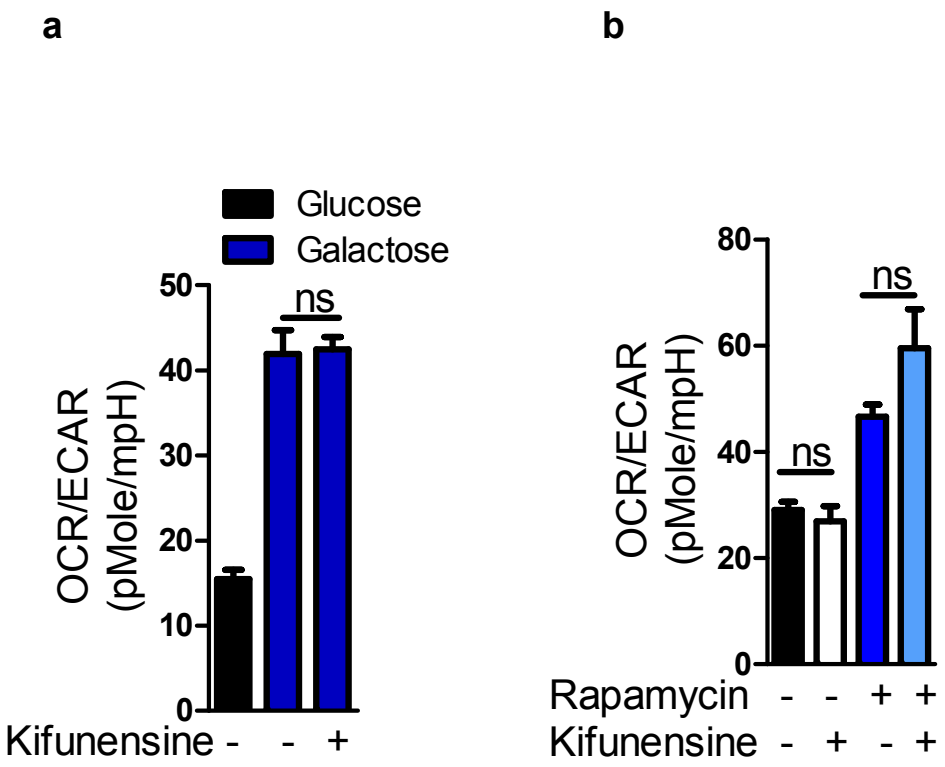

Figure 2.17 Kifunensine does not alter the inhibition of aerobic glycolysis. (a-b) OCR and ECAR were measured by Seahorse of isolated splenic $CD4^+$ T cells activated with anti-CD3/28 under T^H17 inducing conditions for 2 days in (a) Glc and Gal media with and without 10μM kifunensine added at the time of plating, or (b) treated with and without 10μM kifunensine and 1nM rapamycin at the time of plating.

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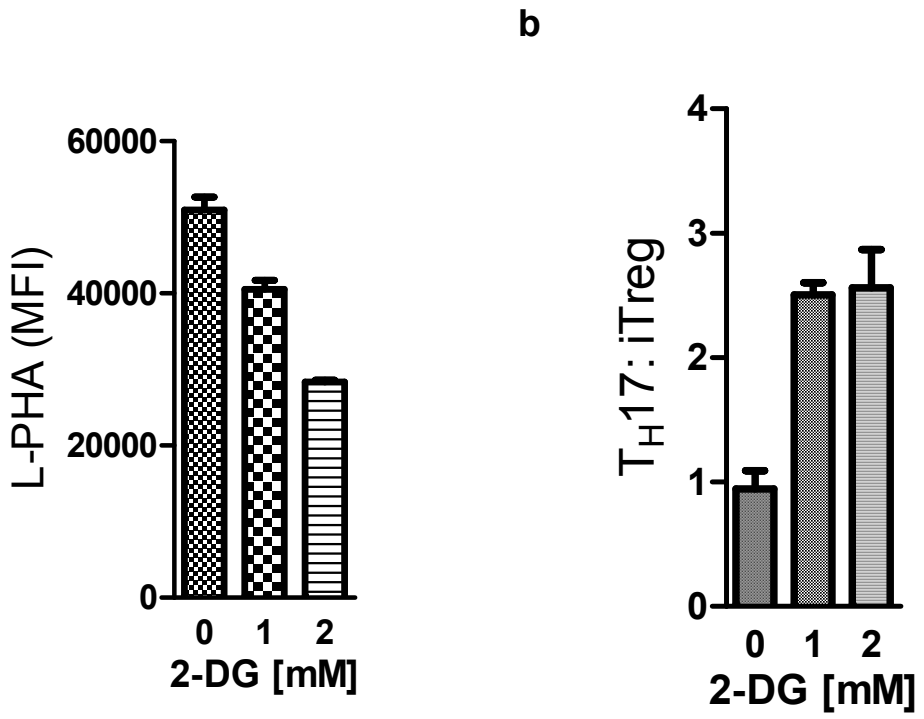

Figure 2.18 2-DG reduces N-glycan branching and promotes T_H17 induction. (a) Extracellular L-PHA was measured on purified $CD4^+$ T cells under T_H17 inducing conditions with and without (0-2mM) 2-DG treatment added at the time of plating and harvested at day 4. (b) Intracellular IL-17 and Foxp3 were measured in $CD4^+$ T cells activated with anti-CD3/28 for 4 days under T_H17 inducing conditions with and without (0-2mM) 2-DG treatment.

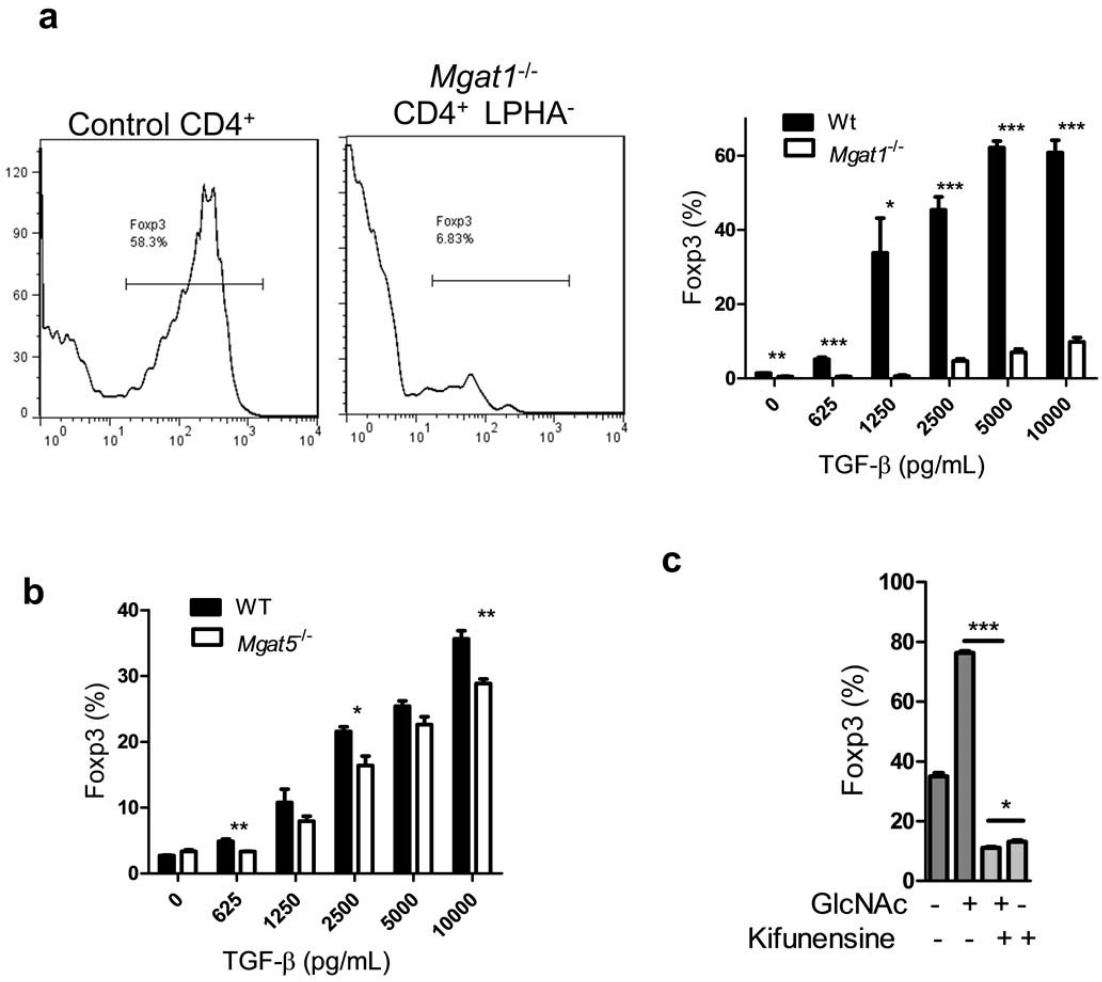

Figure 2.19 Reducing branching decreases iTreg differentiation. (a-c) Flow cytometric analysis of intracellular Foxp3 was measured in isolated splenic $CD4^+$ T cells from (a) Wt and *Mgat1*$^{-/-}$ cells or (b) Wt and *Mgat5*$^{-/-}$ cells and activated with anti-CD3/28 for 4 days in the presence of TGFβ (0-10ng mL^{-1}). (c) Intracellular Foxp3 was measured in isolated splenic $CD4^+$ T cells activated with anti-CD3/28 under iTreg inducing conditions for 4 days with and without 10μM kifunensine added at the time of plating or with or without 40mM GlcNAc supplemented in culture media daily.

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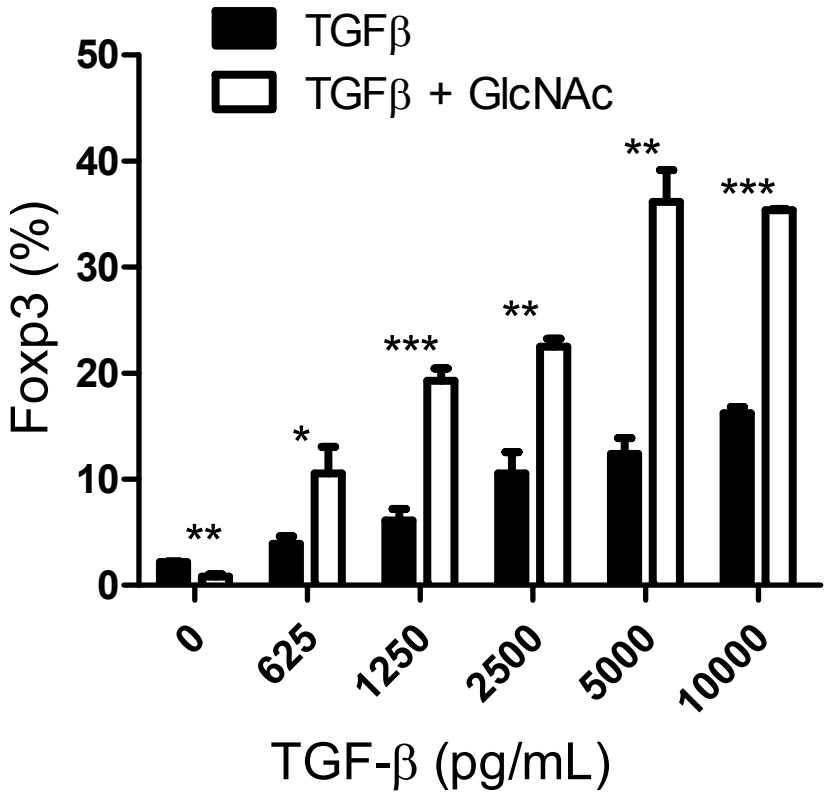

Figure 2.20 Raising branching enhances iTreg differentiation. Flow cytometric analysis of Intracellular Foxp3 was measured in isolated splenic $CD4^+$ T cells activated with anti-CD3/28 for 4 days in the presence of TGFβ alone (0-10ng mL^{-1}) or TGFβ and 40mM GlcNAc supplemented in culture media daily.

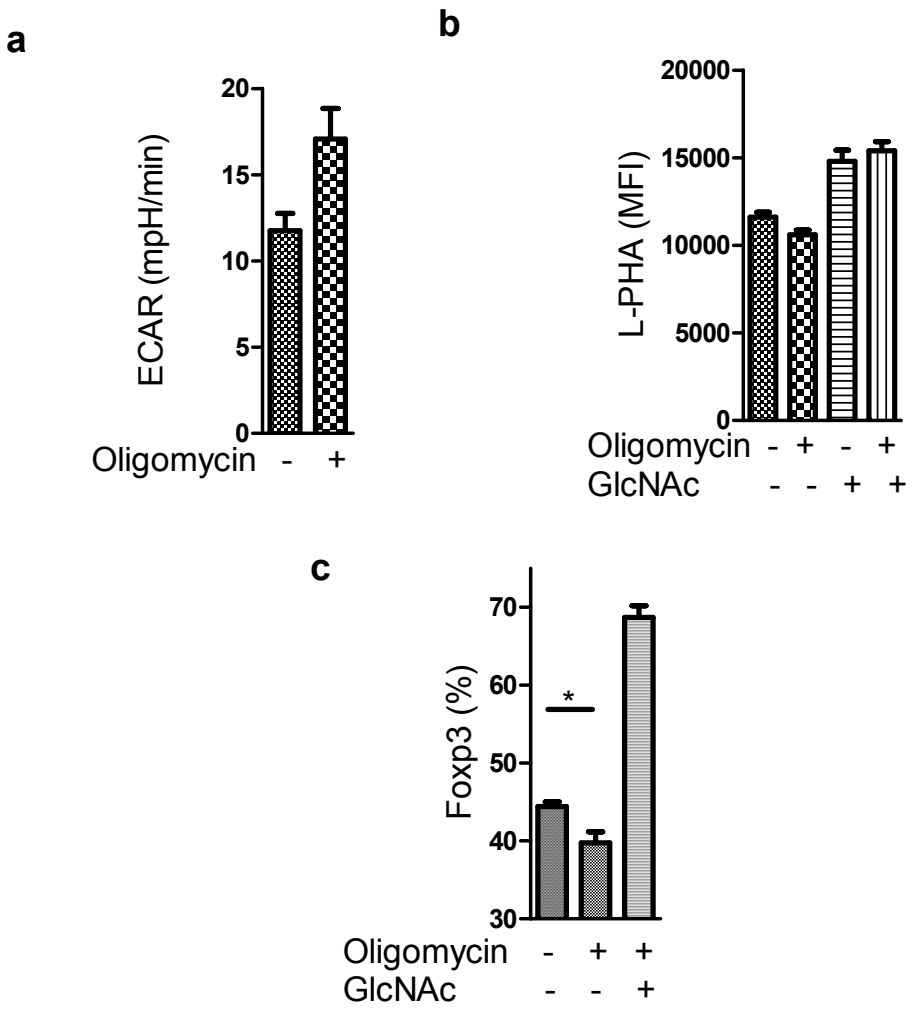

Figure 2.21 Oligomycin increases glycolysis, lowers branching and reduces iTreg differentiation. (a) ECAR was measured by Seahorse in isolated splenic CD4$^+$ T cells activated with anti-CD3/28 for 48 hrs, under iTreg inducing conditions with and without 0.5nM oligomycin treatment. (b) Extracellular LPHA was measured on isolated splenic CD4$^+$ T cells activated with anti-CD3/28 for 2 days under iTreg inducing conditions with or without 0.5nM oligomycin added at the time of plating. (c) Intracellular Foxp3 was measured in CD4$^+$ T cells activated with anti-CD3/28 for 3 days under iTreg inducing conditions, with and without 0.5nM oligomycin added at the time of plating and with or without 40mM GlcNAc treatment supplemented in culture media daily.

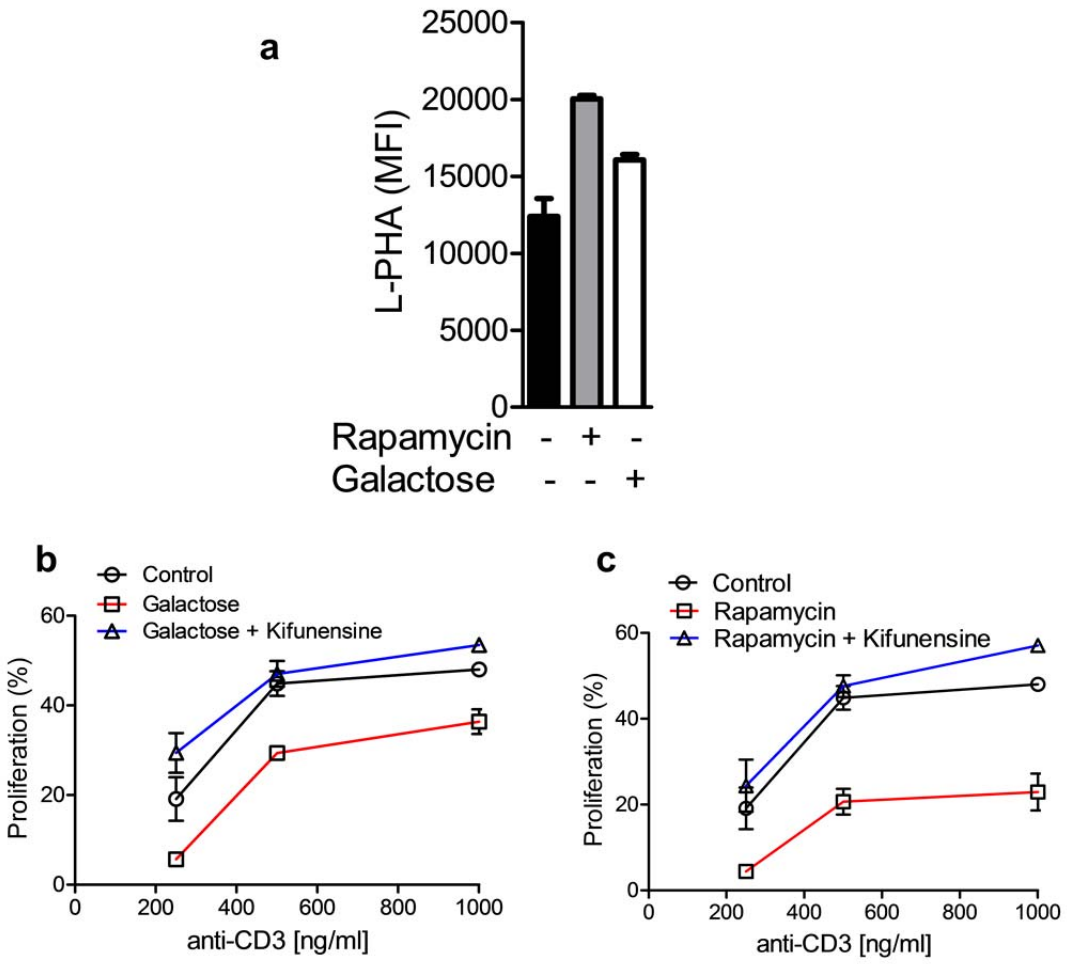

Figure 2.22 Glycolytic inhibition in neutral T cell blasts raises branching and inhibits growth. (a) Extracellular L-PHA was measured on isolated splenic $CD4^+$ T cells activated with anti-CD3/28 for 3 days with and without Gal media, or with and without rapamycin (1nM). (b,c) CFSE-labeled isolated splenic $CD4^+$ T cells were activated with anti-CD3 and cultured in Gal medium or Glc medium with and without rapamycin (1nM), and kifunensine (10μM). CFSE dilution was measured day 3 following activation.

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Chapter 3

Conclusions and Discussions

Regulation of GFAT

As the competition between PFK and GFAT for fructose-6-phosphate is critical to determining whether increased glucose flux raises or lowers branching, it is important to ask which factors influence GFAT expression. Understanding the metabolic and signaling pathways that drive GFAT expression, and the transcription factors that regulate it are critical to moving forward. A greater understanding of GFAT regulation should allow exploitation as a therapeutic target for inflammatory and/or autoimmune disease by controlling T cell growth and pro-inflammatory versus anti-inflammatory differentiation. Furthermore, other regulators of branching besides GFAT should continue to be investigated as potential targets for immunomodulation, such as the N-gycan branching enzymes. Activity of the branching enzymes may be limited by the amount of UDP-GlcNAc substrate availability and therapeutically targeting both may be additive and/or synergistic. For example, targeted increases in Mgat5 enzyme expression in the presence of GlcNAc supplementation may lead to synergistic increases in branching. Thus modulation of expression in conjunction with metabolic supplementation may be another way to regulate T effector cell differentiation and proliferation.

Warburg effect and N-Glycosylation in cancer

The Warburg effect was first described in cancer cells, where branching is pathologically over-expressed and serves as an essential driver of growth, motility and metastasis by maximizing surface retention of receptor tyrosine kinases and limiting clustering of integrins at focal adhesions [1-5]. Our model predicts that the Warburg

effect promotes the cancer phenotype by driving over-expression of branching. Indeed, GFAT expression has been found to be up-regulated in prostate cancer [6, 7]. Similarly, preliminary analysis by others in the Demetriou lab have observed marked increases in GFAT coupled with decreased PFK in melanoma cells compared to melanocytes. GFAT overexpression will out-compete reduced PFK levels for fructose-6-phosphate to drive branching, similar to what we have seen in neutral T cell blasts. Thus, increases in glucose uptake from the Warburg effect and increased GFAT expression may be a general mechanism in cancer to promote N-glycan branching and the cancer phenotype.

Overexpression of branching is a regulator of growth and motility in cancer. Therefore inhibiting branching with kifunensine should abrogate growth and motility without altering glycolysis. Supporting this notion, it has been found that *Mgat5* deficient mice display an inhibition of tumor growth and metastasis [5]; therefore we would hypothesize treatment with kifunensine would yield similar or possibly a more profound inhibition of growth due to the inhibition occurring upstream of all the Mgat enzymes.

Our model predicts that inhibition of the Warburg effect in cancer cells will reduce branching, cell growth, and motility by decreasing glucose flux to the hexosamine pathway. Providing GlcNAc to cancer cells treated with glycolysis inhibitors should by-pass the decrease in glucose up-take by the cell to restore UDP-GlcANc biosynthesis, cell growth and motility. Positive results in such an experiment would confirm that the Warburg effect promotes tumor cell growth and motility by promoting branching via metabolic flux of glucose into the hexosamine pathway.

Glutaminolysis

Glucose and glutamine are highly abundant nutrients in plasma, and in addition to glycolysis, many rapidly proliferating cells like T cells and tumors also rely on glutaminolysis. Cancer growth and transformation is stimulated by glutamine [8] with glutamine used as an alternative energy source for the cell via breakdown to glutamate and α-ketoglutarate, the latter entering the TCA cycle. The upregulation of glutaminolysis has been found to be mediated by c-Myc, a transcription factor which promotes glutamine uptake and the catabolic process of glutamine [9, 10]. c-Myc has also been found to bind to promoter regions of glutamine transporters *ASC2* and *SN2* and enhance levels of transporter mRNA [10, 11]. The products of glutaminolysis can be used to fuel the TCA cycle while the intermediates being produced can also be used for biosynthesis of amino acids, lipids, and other metabolites for the rapidly proliferating cell.

Importantly, glutamine is also a substrate of GFAT, acting as an amine donor to fructose-6-phosphate, yielding glucosamine-6-phosphate and glutamate. Thus the elevated branching displayed by cancer cells may be a consequence of both increased glycolytic flux of fructose-6-phosphate and increased uptake of glutamine. Indeed, excess glutamine raises N-glycan branching in human leukemia T cells [12]. The breakdown of glutamine to glutamate is usually assigned to the enzyme glutaminase; however GFAT may be as important if not more important than glutaminase in this process. Further exploration of the role of GFAT in glutaminolysis and associated affects on branching are warranted. This may elucidate additional mechanisms controlling T cell function and cancer growth and metastasis.

Summary

It has been over 90 years since Otto Warburg's seminal discovery that cancer cells display increased glucose uptake and a switch from oxidative phosphorylation to aerobic glycolysis [13, 14]. Although the Warburg effect is now known to be a general characteristic of rapidly proliferating cells, its purpose has remained poorly understood and controversial. Our data indicate that the Warburg effect controls T cell growth and pro-inflammatory versus anti-inflammatory differentiation by controlling glucose flux into the hexosamine pathway to alter UDP-GlcNAc synthesis and N-glycan branching. Increases or decreases in branching depend on the relative activities of PFK and GFAT, the two key enzymes directing fructose-6-phosphate into glycolysis versus the hexosamine pathway, respectively. Thus, metabolically altering N-glycosylation is an essential function of the Warburg effect, a result with widespread implications for inflammatory diseases and cancer.

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