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Distinct metabolic pathways mediate regulatory T cell differentiation and function

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Highlights

- Preferential metabolic pathways employed by Tregs *in vivo* and *ex vivo* differ from *in vitro*-induced Tregs.
- Metabolic ‘harshness’, particularly the deterioration of glycolysis, positively affects Treg differentiation and function, while negatively correlating with Treg clonal expansion and migratory capacity.
- These context-dependent findings provide new insights into the behaviour of Tregs with implications for both tumour immunology and autoimmunity.

Abstract

Investigation of the cellular metabolic pathways of immune cells, or immunometabolism, is a field of increasing interest. An understanding of immunometabolism provides routes to modifying T cell function for therapeutic purposes. Here, we review immunometabolism with a specific focus on regulatory T cells (Tregs). While T cells are known to switch their metabolic profile from oxidative phosphorylation to aerobic glycolysis upon activation, *in vitro*-induced Tregs display alternate metabolic characteristics which may be related to their specialised suppressive function. Recent data suggest that the preferential pathways employed by Tregs differ *in vivo* and *ex vivo*. Metabolic ‘harshness’, particularly the deterioration of glycolysis, positively affects Treg differentiation and function, while negatively correlating with Treg clonal expansion and

migratory capacity. These context-dependent findings provide new insights into the behaviour of Tregs with implications for both tumour immunology and autoimmunity. This review examines the field in detail, offering an overview of our current understanding of Treg immunometabolism.

Abbreviations

2-HG	2-hydroxyglutarate
AA	Amino acid
ACC	Acetyl-CoA carboxylase
Acetyl-CoA	Acetyl-coenzyme A
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
AU	Adenylate-uridylate
CNS	Conserved non-coding sequence
CPT1	Carnityl-palmitate transferase 1
EAA	Essential amino acid
FAO	Fatty acid oxidation
FAS	Fatty acid synthesis
FOXO	Forkhead box O
FoxP3	Forkhead box P3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCK	Glucokinase
Glut1	Glucose transporter 1
GLS	Glutaminase
HDAC	histone deacetylase
HIF-1 α	Hypoxia-inducible factor 1 α
IDO	Indoleamine 2,3-dioxygenase
IFN- γ	Interferon-gamma
IL-6	Interleukin-6
iTreg	in-vitro induced Treg
KO	Knock-out
LNAA	Large neutral amino acid
mTOR	Mammalian target of rapamycin

mTORC	mTOR complex
OXPHOS	Oxidative phosphorylation
PHD	Prolyl hydroxylase domain-containing enzyme
PI3K	Phosphoinositide 3-kinase
PPP	Pentose phosphate pathway
PTEN	Phosphatase and tensin homolog
pTreg	Peripherally-derived Treg
ROS	Reactive oxygen species
TCA cycle	Tricarboxylic acid cycle
Tconv	Conventional T cells
TCR	T cell receptor
TGF- β	Transforming growth factor-beta
Th1	Helper T cell type 1
Th17	Helper T cell type 17
TLRs	Toll-like receptor
Treg	Regulatory T cells
TSDR	Treg-specific demethylation region
tTreg	Thymus-derived Treg
UTR	Untranslated region
VHL	Von Hippel-Lindau protein
WT	Wild type
α -KG	α -ketoglutarate

Keywords: Regulatory T cells; Immunometabolism; Tolerance; Hypoxia; mTOR

Introduction

Metabolism is a fundamental biological process producing the adenosine triphosphate (ATP) required for cell maintenance and growth. The net metabolic expenditure of a cell is a balance between anabolic pathways (energy-dependent macromolecular biosynthesis) and catabolic pathways (energy-producing macromolecular degradation).

Catabolism can be divided into two main pathways: glycolysis, a rapid but inefficient production of ATP from glucose; and oxidative phosphorylation (OXPHOS), in which intermediates from glucose, fatty acids and amino acids enter the oxygen-dependent tricarboxylic acid (TCA) cycle and electron transport chain in mitochondria (Figure 1). The concept that certain cell populations may have metabolic predilections first came from Otto Warburg who observed that oncologic cells preferentially used glycolytic metabolic pathways instead of OXPHOS even in conditions with sufficient oxygen, termed 'aerobic glycolysis' (1). This was subsequently observed in leukocytes, which also preferentially use aerobic glycolysis upon activation (2). These observations gave birth to the field of immunometabolism.

The immunometabolic pathways resulting in conventional T cell activation and differentiation have naturally been the focus of much study. It is well established that conventional T cells activated by T cell receptor (TCR) binding alongside CD28 co-stimulation switch their metabolism from oxidative phosphorylation to glycolysis independent of oxygen availability (3, 4). This ensures rapid availability of ATP (5), and a pool of catabolic intermediates that can supply other anabolic pathways including the pentose phosphate and serine pathways. These pathways provide a carbon source for the macromolecular synthesis required for basic cellular processes such as clonal expansion (6, 7). In addition to the catabolic and anabolic importance of glycolysis, T cell metabolism also modulates their effector functions either by directly regulating cytokine synthesis, by indirectly modulating differentiation of CD4⁺ T cell subsets, or by altering T cell activation. For instance, the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has a further function inhibiting interferon- γ (IFN- γ) mRNA translation (8). Upregulation of glycolysis following TCR activation results in GAPDH being engaged preferentially in metabolic pathways and therefore prevents its inhibitory function on IFN- γ mRNA translation (8). The mechanism of GAPDH-mediated post-transcriptional modification is through binding to adenylate-uridylate (AU) rich elements within the IFN- γ mRNA 3' untranslated region (UTR). Since AU-rich elements are also present within mRNA coding regions of other effector cytokines such as IL-4 and IL-17 α , it may be that GAPDH has a more general role in regulating cytokine production (9). Glycolysis has been further implicated in the maintenance of Tconv effector function through modulation of calcium signaling: when Tconv cells are activated in a glucose-

sufficient microenvironment, the glycolysis intermediate phosphoenolpyruvate suppresses sarco/endoplasmic reticulum calcium-ATPase (SERCA) activity, thereby maintaining TCR-mediated calcium-NFAT1 signaling (10). Glycolysis also modulates cytokine production through epigenetic modification. Histone acetylation status is known to modulate gene transcription, with acetylation associated with transcriptional activation and deacetylation associated with transcriptional repression (11). Citrate, a TCA cycle intermediate, can be transported to the cytosol where it becomes acetyl-CoA. This promotes histone acetylation of the gene coding IFN- γ . A metabolic switch to aerobic glycolysis promotes citrate production at the expense of TCA cycle activity, therefore enhancing cytosolic acetyl-CoA accumulation and resulting in enhanced IFN- γ transcription (12). CD4⁺ T cell differentiation is equally impacted by the availability of metabolites. For example, high extracellular glucose promotes T helper 17 (Th17) instead of T helper 1 (Th1) differentiation through the production of reactive oxygen species (ROS) (13). ROS generation through restriction of glutaminolysis, however, seems to have the opposite effect on Th1/Th17 differentiation demonstrating the complexity of these relationships (14).

Regulatory T cells (Tregs), defined by expression of the master transcription factor forkhead box P3 (FOXP3), are crucial for maintaining immune homeostasis (15-18). This is illustrated by the rare life-threatening genetic condition Immunodysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome, caused by an inactivating *FOXP3* mutation that results in impaired Tregs and the subsequent development of various autoimmune pathologies (16, 19). Tregs can be divided into thymus-derived Tregs (tTregs) generated from thymocytes during T cell maturation and peripherally-derived Tregs (pTregs) which differentiate from conventional T cells (Tconvs). Tregs can also be produced *in vitro* from Tconv, in which case they are called *in vitro*-induced Tregs (iTregs) (20).

There is intense interest in the intersection between immunometabolism and immune tolerance. Delineating the contribution of metabolism to Treg induction and function offers the exciting prospect of modulating these for therapeutic purposes. Furthermore, recent work has challenged previous data positioning OXPHOS as the dominant metabolic pathway within Treg, instead suggesting more nuanced metabolic

contributions to Treg differentiation and function (21). This review will present an overview of recent developments in Treg metabolism focussing on the commonalities and differences between Treg differentiation and function.

Metabolic control of Treg differentiation

Amino acid metabolism

Upon TCR-mediated T cell activation with appropriate co-stimulation, T cells undergo clonal expansion and differentiate into different functional subsets. The compartment into which an individual cell ultimately enters is based on numerous subsequent signals, such as the relative and specific cytokine abundance during differentiation. For example, it is well known that Treg differentiation is driven *in vitro* and *in vivo* by the presence of TGF- β which signals to the conserved non-coding DNA sequence 1 (CNS1) in *Foxp3* (22-25). However, the co-existence of the inflammatory cytokine interleukin-6 (IL-6) skews differentiation away from Tregs towards pro-inflammatory Th17 cells (26-28). In addition to cytokine signals, several metabolic pathways are also implicated in Treg differentiation (Figure 2).

Tryptophan is an essential amino acid (EAA) that, by definition, cannot be synthesised *de novo* in humans. Of all essential amino acids, tryptophan is present in the lowest concentration in humans and therefore in starvation states is the first to be depleted. Initially, the role of tryptophan catabolism in Treg function was primarily thought to be mediated indirectly via the indoleamine 2,3-dioxygenase (IDO) activity of other cells (27). However, more recently the availability of EAA has been demonstrated to impact Treg differentiation directly: EAA deprivation synergises TGF- β such that Treg differentiation occurs at TGF- β concentrations that would be insufficient in metabolically replete conditions (29).

Slc7A5 is a large neutral amino acid (LNAA) transporter with substrates including leucine and methionine. Blocking LNAA uptake via Slc7A5 strongly inhibits Th1 and Th17 differentiation (30). However, blocking Slc7A5 does not affect Treg differentiation. This supports distinct metabolic pathways driving differentiation of Tregs and Tconv. Under

EAA-deprived conditions, mechanistic target of rapamycin (mTOR) complexes have been implicated in Treg differentiation (29, 30). TCR-mTOR pathways negatively regulate Treg differentiation via antagonising smad3 and smad4, members of the smad protein family phosphorylated during TGF- β signaling, or via inactivating Foxo1 and Foxo3, members of the forkhead box O (FOXO) family of transcription factors. Conversely, mTOR inhibition enhances Treg differentiation (31-34). Importantly, the activation of mTOR complex 1 (mTORC1) is directly regulated by amino acid availability in the extracellular environment (35, 36). There is also preliminary evidence suggesting that activation of mTOR complex 2 (mTORC2) is regulated by the same mechanism (37). Indeed, both EAA deprivation and Slc7A5 knock-out T cells exhibit reduced phosphorylation of S6, a protein downstream of mTORC1, mirroring that seen in culture with the mTOR inhibitor sirolimus (29, 30).

Glutamine deprivation also promotes Treg differentiation via mTORC1 inhibition (38, 39). Interestingly, even in Th1 polarising conditions T cell differentiation is skewed from Th1 to Treg by glutamine deprivation. Addition of α -ketoglutarate (α -KG), a glutaminolysis product, restores the initial Th1 polarisation indicating that glutaminolysis regulates Th1 and Treg differentiation via mTOR activation even within a pro-inflammatory cytokine milieu (39). Furthermore, glutamine metabolism also regulates Treg differentiation through epigenetic modulation. α -KG is a substrate for 2-hydroxyglutarate (2-HG), which increases methylation of the Treg-specific demethylated region (TSDR) within the FOXP3 locus, thereby inhibiting Treg differentiation (40). However, deletion of glutaminase, a key enzyme required for glutaminolysis, does not alter Treg differentiation *in vitro* nor Treg numbers *in vivo*, despite glutaminase deletion effectively reducing intracellular α -KG (14). This implies that the glutamine deprivation that enhances Treg differentiation is not necessarily only due to diminished glutaminolysis.

Fatty acid metabolism

Energy starvation causes adenosine monophosphate-activated protein kinase (AMPK) activation, which is known to have an opposing role to mTOR dependent pathways (41). The influence of AMPK signaling in Treg differentiation was first described by data showing increased *in vivo* Treg differentiation in mice treated with the AMPK activator metformin (42). Although the mechanism by which metformin activates AMPK is not completely clear (43), another study conclusively proved that Treg differentiation can be

enhanced using a directly-activating AMP analogue (44). Because AMPK is known to enhance fatty acid oxidation (FAO), both groups further investigated the relationship between Treg differentiation and FAO using the carnityl-palmitate transferase 1 (CPT1) inhibitor, etomoxir. Etomoxir is known to inhibit a rate-limiting enzyme in long chain FAO and has been shown to abrogate Treg differentiation (42, 44). However, many reports have highlighted the off-target effects of etomoxir at standard therapeutic concentrations (45-47). As such, the prevention of Treg differentiation using etomoxir observed in these studies may not necessarily be due to FAO inhibition. Indeed, neither low dose etomoxir that is sufficient to inhibit CPT1, nor *CPT1* deletion, have an effect on Treg differentiation (47).

Conversely, AMPK-mediated inhibition of fatty acid synthesis (FAS) appears to have an important role in Treg polarisation. Acetyl-coA is required for FAS, and can be impaired by Soraphen A, an acetyl-CoA carboxylase (ACC) inhibitor, or 4EGI-1, an inhibitor of ACC translation that blocks the interaction between eIF4E-eIF4G. Both Soraphen A and 4EGI-1 decrease Th17 and increase Treg differentiation, even under Th17-polarising conditions (48, 49). ACC has two main isoforms: ACC1 is highly lipophilic, localises to the cytoplasm, and catalyses an intermediate required for FAS; and ACC2 which is lipophobic localises to mitochondria and catalyses an intermediate required for FAO (50). Investigating CD4⁺ T cell-specific ACC1 and ACC2 knock-out (KO) mice revealed that ACC1, but not ACC2, KO enhances Treg differentiation (48). In addition, exogenous long-chain fatty acid abrogates the effects of ACC inhibition. This indicates that ACC inhibition enhances Treg differentiation through FAS inhibition. However, the mechanism by which FAS controls Treg/Th17 differentiation remains to be defined.

The importance of fatty acids for Treg differentiation is also well-illustrated by the microbiome and metabolome of the intestinal tract. Bacteria-derived short-chain fatty acids, especially butyrate, are one of the key promoters of colonic Treg differentiation (51-53). In addition to skewing dendritic cells towards a tolerogenic phenotype, butyrate can also work directly on T cells as a histone deacetylase (HDAC) inhibitor, either by epigenetically modifying *Foxp3* expression or directly stabilising the Foxp3 protein by acetylation (51, 53, 54). Extracellular short-chain fatty acids may also decrease glycolytic rate which reduces glycolysis-FAS flux therefore promoting Treg induction by lowering

de novo FAS.

Glucose metabolism and hypoxia

As AMPK enhances Treg differentiation, it may be assumed that limiting glucose availability or hypoxic conditions could promote Treg differentiation by increasing the AMP/ATP ratio. This is indeed observed in T cells, where polarisation with small molecule glycolysis inhibitors or under hypoxic conditions enhances Treg differentiation (55-59). However, it is important to note that these enhancements are largely context dependent. Constitutive degradation of hypoxia-inducible factor 1 α (HIF-1 α) is rapidly inhibited during severe hypoxia which promotes several cellular responses including modulating metabolic pathways to adapt to hypoxic conditions (60). Interestingly, even under normoxic conditions, activated T cells utilise HIF-1 α signaling to switch to glycolytic pathways via mTORC1 (58, 61). Furthermore, HIF-1 α has an essential role in promoting the differentiation of Th17 subsets under Th17 polarising conditions, although does not effect Treg differentiation under Treg-polarising conditions (58, 62). Glycolysis inhibition restores the skew from Th17 cells back towards Tregs (55, 58). A mechanistic description of how glycolysis causes Th17 polarisation remains to be defined, and could perhaps be mediated by FAS. The enhanced Treg differentiation observed under hypoxic conditions is completely abolished by adding IL-6 *in vitro* (56, 62). The balance of Treg/Th17 differentiation under hypoxic conditions is therefore completely context dependent: hypoxia itself appears to enhance Treg differentiation in Treg polarising conditions, probably via AMPK signaling, but in an inflammatory milieu, hypoxia promotes Th17 cell differentiation through a HIF-1 α -mediated glycolysis dependent mechanism.

Intracellular metabolic activation and extracellular metabolic provision clearly affect Treg differentiation (Figure 2). As outlined above, several distinct metabolic pathways underpin these characteristics, particularly mTOR and AMPK signaling, although the extracellular conditions in which T cells proliferate also significantly contribute. Viewing immunometabolism from the perspective of Treg suppressive function may reveal exploitable immunometabolic differences which will be considered in the following section.

Metabolic control of regulatory T cell suppressive function

The mTORC1-HIF1 α axis

Treg induction is enhanced by low mTOR signalling which reduces the glycolysis-dependent metabolic phenotype. As expected, genetic deletion of mTORC1 enhances iTreg differentiation (31, 34). However, mTORC1-deleted tTreg cells have impaired suppressive capacity *in vitro* that translates into a scurfy-like phenotype *in vivo* (63, 64). The impaired Treg function from mTORC1 deletion is due to the loss of metabolic homeostasis in TCR-activated Tregs reflected by reduced production of glucose-derived lipids and nucleotides, and downregulated expression of mitochondrial genes (64, 65). Differing metabolic requirements between iTreg induction and tTreg function can also be observed in Tregs in conditions of EAA deprivation. The GTPases Rag and Rheb have a central role sensing the availability of amino acids required for maintaining mTORC1 activation in Treg. Treg specific deletion of Rag or Rheb in mice causes a scurfy phenotype (66). It therefore appears that tTreg require extracellular EAA for activation-induced mTORC1 signalling which maintains the metabolic homeostasis of fully functional Treg.

Even though mTORC1 signalling is fundamentally required for tTreg metabolic homeostasis, hyperactivation of mTORC1 signalling appears to have deleterious effects on tTregs. tTregs, in contrast to Tconvs, have increased levels of phosphorylation of mTORC1 and pharmacological inhibition of mTORC1 with sirolimus positively affects Treg proliferation (in contrast to mTORC1 gene KO) (64, 67). Notably, sirolimus enhances previously-activated Treg suppressive activity (68). On the other hand, hyperactivation of mTORC1 through Toll-like receptor (TLR) stimulation or deletion of TRAF3IP3, a negative regulator of mTORC1, decreases Treg suppressive function in addition to augmenting the rate of aerobic glycolysis (69, 70). Importantly, Tregs that express high levels of the glucose transporter 1 (Glut1), one of the genes upregulated by mTORC1-HIF-1 α signalling, lose their suppressive function (69). Moreover, Glut1 KO Tregs are not functionally impaired, in contrast to Tconv subsets (71). This indicates that Glut1-mediated glycolytic flow negatively regulates Treg function. A delicate balance of mTORC1 signalling is therefore required for optimal Treg function: too high negatively impacts Treg effector functions, at least partially due to the upregulation of glucose metabolism via Glut1 expression; too low impairs the homeostatic balance.

Apart from hypoxia, HIF-1 α expression is also induced by TCR-mTORC1 signalling. Akin to mTORC1, overexpression of HIF-1 α also negatively regulates Treg function. HIF-1 α KO Treg have unimpaired suppressive function under normoxic conditions *in vitro*. However, surprisingly, HIF-1 α KO Treg demonstrate significantly enhanced suppressive function compared to wild type (WT) Tregs under hypoxic conditions (72). This enhanced suppressive function is completely abrogated by pharmacological inhibition of the mitochondrial pyruvate carrier causing reduced energy production from glucose-derived OXPHOS, and gradually compensated for by increasing the glycolytic rate. This suggests that either simple down-regulation of respiration, direct enhancement of glycolytic flow, or a combination of both may be key mechanisms explaining the enhanced suppressive function of HIF-1 α KO Treg. In agreement with this *in vitro* study, hyperactivation of HIF-1 α using deletion of the E3-ubiquitin ligase tumour suppressor gene von Hippel-Lindau (VHL) or Deltex-1 which both contribute HIF-1 α degradation almost completely abolishes Treg suppressive function *in vivo* (73, 74). These studies conclusively demonstrate that HIF-1 α hyperactivation negatively regulates Treg suppressive function by enhancing glycolysis.

More recently, a role for HIF-2 α in Treg function has been reported (75). In this study, PHD2-silenced Tregs lost their suppressive capacity *in vivo*, an effect which was reversed by concomitant HIF-2 α silencing. Similarly to HIF-1 α , stabilisation of HIF-2 α can also be promoted by the mTOR pathway (76). From the metabolic aspect, in contrast to HIF-1 α which directly enhances glycolysis, HIF-2 α signalling can enhance glycolysis indirectly by suppression of FAO (77). Thus, there is evidence that both HIF-1 α and HIF-2 α negatively regulate Treg function, at least partially through the enhancement of glycolysis.

Although a supra-physiologic concentration of HIF-1 α impairs Treg suppressive function *in vivo*, there are conflicting results from studies investigating the importance of HIF-1 α in Tregs *in vivo*. For example, HIF-1 α KO Treg lose their suppressive capacity in a T cell-mediated mouse model of colitis (56). Interestingly, tumour infiltration of intravenously injected HIF-1 α KO Tregs into a glioma tumour in mice is significantly impaired (72), with similarly impaired migration demonstrated using *in vitro* trans-well migration assays. This suggests that HIF-1 α is required for Treg migratory capacity, a potential explanation

for differing *in vitro* and *in vivo* data. Taken together, HIF-1 α is important to maintain Treg migratory capacity into peripheral tissues, whilst over expression of HIF-1 α under hypoxic conditions impairs Treg suppressive function.

The mTORC2-FOXO1 axis

The improved survival observed in mTORC1 and 2 double-KO Tregs compared with mTORC1 KO Treg gathered significant interest by positioning mTORC2 as a negative regulator of Treg function (64). This was further confirmed by the spontaneous development of lymphoproliferative diseases in a Treg-specific phosphatase and tensin homolog (PTEN) KO mouse model (78). PTEN is a negative regulator of phosphoinositide 3-kinase (PI3K) which acts upstream to prime both mTOR pathways. In the PTEN KO mouse model, Tregs demonstrate selective over-activation of mTORC2 but not of mTORC1. Interestingly, Tregs in this model are hypermetabolic with a significant increase in glycolysis (78). In addition to its other important roles, FoxP3 also acts as a metabolic regulator of Tregs through suppressing glycolysis. Recent work has revealed enhanced mTORC2 but not mTORC1 phosphorylation in dysfunctional Tregs from patients with IPEX. In these patients, treatment of Tregs with an mTOR inhibitor restores this hypermetabolic state and dramatically recovers their suppressive function *in vitro* (79). This indicates that the dysfunction observed in Foxp3-mutated Tregs is at least in part due to metabolic dysregulation with overactivation of the mTORC2 pathway.

The relationship between mTORC2 overactivation and Treg dysfunction has also been observed in a scurfy mouse model. Here, selective KO of mTORC2 in Tregs with mutated *Foxp3* ameliorates IPEX. Metabolic phenotyping of these Foxp3-deficient Tregs reveals a significantly increased glycolysis rate and increased percentage of glucose consumption by OXPHOS compared to WT Treg in which fatty acids are the main source for OXPHOS. *PFKFB3* encodes 6-phosphofructo-2-kinase/fructose-2,6- biphosphatase 3 which catabolises fructose 6-phosphate into fructose 2,6-biphosphate—an important activator of the rate limiting enzyme phosphofructokinase-1 required for glycolysis. Deletion of *PFKFB3*, and direct pharmacological inhibition of glycolysis with the glucose analogue 2-deoxy-glucose restores Treg suppressive function in Foxp3 mutated mice *in vitro*. This demonstrates an important link between Foxp3 and Treg glycolytic metabolism required for functional Treg (79). It has also been demonstrated that

mTORC2 hyperactivation in Foxp3 mutated Treg reduces the expression of Foxo1. Furthermore, Foxo1 deletion impairs rescue from IPEX in mTORC2 KO. This suggests that the mTORC2-Akt-Foxo1 axis is a key regulator of Treg function (79). Importantly CD4-specific Foxo1 KO mice develop a spontaneous autoimmune phenotype characterised by dysfunctional Tregs, and Treg-specific Foxo1 KO mice develop a fatal IPEX-like syndrome (80, 81). This positions Foxo1 as a crucial regulator required for maintenance of Treg suppressive function through the control of metabolism.

However, overexpression of Foxo1 also impairs Treg suppressive function *in vivo* through inhibiting their migratory capacity. Mice with CD4⁺ cells expressing a mutated form of Foxo1, which are therefore resistant to mTORC2-Akt triggered inhibition, also develop an autoimmune phenotype (82). An autoimmune phenotype is also observed in mice overexpressing Treg-specific Foxo1. Tregs in these mice have impaired capacity to migrate into peripheral tissues and therefore accumulate in secondary lymphoid organs (83). This suggests a pivotal role for Foxo1 inactivation in Treg migration. An important role for mTORC2-mediated expression of glucokinase (GCK), which directly binds to actin to supply the energy source for cytoskeleton modification via glycolysis, has also been described in humans and mice (84). Whether GCK expression is mediated by Foxo1 or other mechanisms is yet to be determined. However, clearly, mTORC2 signaling is important to maintain Treg migratory capacity through inhibition of Foxo1.

In summary, mTORC2 inhibition of Foxo1 negatively regulates Treg suppressive function by enhancing the cellular metabolic rate, driven largely by glycolysis. However, glycolysis mediated by mTORC2 expression has an important role for successful Treg migration into peripheral tissues.

Lkb1 and lipid metabolism

Although AMPK signalling enhances iTreg differentiation, Treg-specific AMPK deletion does not result in phenotypic alterations (85, 86). However, Treg-specific deletion of *Lkb1*, an upstream kinase that activates AMPK, causes mice to develop a scurfy-like phenotype with significantly lower Treg survival and impaired suppressive function. This demonstrates a specific role for Lkb1 signalling independent to that of AMPK (85-87).

Deletion of Bim, a proapoptotic factor, would be expected to reduce cell death. Indeed, mice carrying *Lkb1* KO Tregs with Bim deletion have normal numbers of circulating Treg and yet still all suffer from catastrophic systemic autoimmune diseases. This suggests that *Lkb1* KO does not simply cause high rates of Treg death (86). Metabolic analysis of these *Lkb1* KO Treg reveals significantly decreased lipid synthesis (85-87). Mice with Treg-specific deletion of HMG-CoA reductase, required in a rate limiting step of the mevalonate pathway in lipid synthesis, also develop systemic autoimmune disease (88). Furthermore, pharmacological inhibition of lipid synthesis with a HMG-CoA reductase inhibitor dramatically reduces Treg suppressive function in both *in vivo* and *in vitro* models (64, 87). Moreover, supplementation of mevalonate to Treg-specific *Lkb1* KO mice significantly ameliorates the autoimmune phenotype (87). These studies reveal an important role for *Lkb1*-mediated cholesterol synthesis in the maintenance of tTreg function.

Fatty acid oxidation is required to maintain Treg mitochondrial homeostasis (89, 90). CD36, an extracellular fatty acid receptor highly expressed on intratumoral Treg, enables metabolic adaptation to microenvironments with high lactic acid concentrations via peroxisome proliferator-activated receptor β (PPAR- β) signaling (89). Similarly, fatty acid binding protein 5 (FABP5), an important intracellular chaperone, is necessary for mitochondrial integrity, with disruption of extracellular fatty acid utilization resulting in impaired Treg mitochondrial homeostasis (90). However, acute mitochondrial disruption by FABP5 inhibition causes mitochondrial DNA release into cytoplasm, which enhances Treg suppressive function through promoting IL-10 production (90).

Conclusions and future perspectives

It is clear that the metabolic pathways underlying Treg differentiation and Treg function are separate (Table 1). In general, Treg differentiation is enhanced by metabolically 'harsh' conditions such as low EAA, glucose and oxygen availability (Figure 2). Even though epigenetic regulation of Treg differentiation through α -KG-derived 2-HG has been described, mechanistically, inhibition of mTOR signalling resulting from low amino acid availability, alongside antagonism of AMPK activated by low cellular ATP, seem to have a key role in enhancing Treg differentiation. mTOR has two functions here: firstly it

directly regulates Treg differentiation by antagonising smad3 and 4 signalling, and secondly it induces Tconv differentiation. For example, mTOR signalling is important for Th17 differentiation through the mTOR-HIF-1 α axis, resulting in high glycolytic flux and subsequent FAS. Therefore, AMPK activation under hypoxic conditions preferentially induces Tregs whilst hypoxia-induced HIF-1 α signalling makes T cells more glycolytic causing preferential induction of Th17 cells. There are interesting parallels here to TGF β . Available evidence suggests that hypoxia-enhanced Treg differentiation is largely context-dependent, directed by other factors including IL-6 and nutrient availability. Even in pro-inflammatory conditions favouring Tconv differentiation, Treg differentiation is still induced by disruption of the mTOR-HIF1 α -glycolysis axis through either restricted external amino acid availability, genetic HIF-1 α deletion, or direct inhibition of glycolysis. This indicates that metabolism is a key mechanistic element in CD4 T cell differentiation.

Unlike glycolysis and glutaminolysis, fueling by fatty acids do not hinder Treg differentiation. Although due consideration must be given to the off-target effects of etomoxir, the enhancement of FAO by FoxP3 indirectly suggests that Treg energy homeostasis maintained by fatty acids can also be important to induce Treg differentiation, at least partially by maintaining low throughput from other metabolic pathways including glycolysis (91). Furthermore, microbiome derived short-chain acids, such as butyrate, may potentially possess a dual function both in fueling FAO and as a HDAC.

When it comes to the immunometabolism of Treg function, further work is required to definitively establish the relationship between metabolic 'harshness' (especially low glucose availability) and Treg suppressive function. This complexity in part arises from the differing metabolic demands of Tregs depending on anatomical location (Figure 3). Originally, a clear contrast between Treg differentiation and Treg function appeared to emerge from the description of Treg-specific mTORC1 KO mice which suffered from catastrophic autoimmune disease. The primary function of mTORC1 signaling is to maintain the metabolic fitness of Tregs upon activation. In fact, activated naïve Tregs first undergo clonal expansion which is undoubtedly a metabolically demanding anabolic process. Presumably, glycolysis and glutaminolysis enhancement to meet these anabolic demands also requires appropriate mTORC1 activation. In addition to mTORC1 signaling,

recent studies have also described the importance of the Lkb1 pathway to maintain lipid synthesis, most likely from glycolysis.

The importance of mTORC1- and mTORC2-maintained glycolysis in enabling successful Treg migration from secondary lymphoid organs into peripheral tissues is also apparent. There are interesting contrasts between the hexokinase isotypes preferred by Tconv (HK1, induced by mTORC1) and Tregs (GCK, induced by mTORC2) (84, 92). mTORC1 signaling may also be required for Treg migration through activating HIF-1 α , perhaps by increasing the expression of glycolytic enzymes such as lactate dehydrogenase A. This allows a readily available energy supply for GCK to bind actin required for cytoskeleton rearrangement and cell migration.

However, high glycolytic flow negatively regulates Treg function in peripheral tissues. *FOXP3*, the Treg master gene, directly maintains Treg suppressive function. Interestingly, it also works as a regulator of Treg metabolism by down regulating glycolysis (69, 91, 93). As described above, mTORC2 KO or direct inhibition of glycolysis in FoxP3-mutated Tregs partially recovers their suppressive function, illustrating the metabolic function of FoxP3. However, whether FoxP3 directly antagonises the mTORC2 pathway or produces its metabolic functions indirectly by other mechanisms remains to be elucidated. Surprisingly, utilisation of glycolysis under hypoxic conditions (anaerobic glycolysis) to compensate for energy deprivation also reduces Treg suppressive function. As such, the precise mechanism by which glycolysis impairs Treg suppressive function requires further study.

Insight into the immunometabolism of Tregs has evolved dramatically this decade. However, often immunometabolic studies have focused on the cellular level. The effects of systemic metabolic dysregulation on Treg induction and function therefore remains unclear. For example, the concentration of insulin in peripheral blood increases as a result of modern high calorie diets or insulin resistance of peripheral tissues. It was recently revealed that insulin boosts Tconv effector functions via enhancing Tconv metabolism (94), and perhaps insulin receptor resistance could also be a feature of T cells. On the other hand, a low-calorie ketogenic diet produces ketone bodies, a substitute fuel for glucose. Interestingly, extrahepatic ketone body production is largely

derived from the conversion of microbiome-derived butyrate (95). Although low glucose availability and high butyrate in the local environment can preferentially induce Tregs, whether the ketone bodies produced by both conditions affect Treg induction is not known. Viewing Treg metabolism at a systemic level therefore offers the potential to identify generic immunometabolic pathways that may mediate immune regulation in disparate regulatory cell populations.

Finally, it is worth highlighting that the majority of Treg immunometabolic studies have used mouse cells and, as such, fundamental differences between the immunometabolism of human and mouse Tregs may be obscured. For example, culture of human T cells with a glycolysis inhibitor causes impaired Treg polarisation (96), in contrast to the mouse studies described. In this study, Tregs were polarised with suboptimal TCR stimuli and without TGF- β . As such, the activated human T cells in this setting may have had significantly lower mTOR signaling and glycolytic flow, causing preferential Treg differentiation. Interestingly, recent mouse studies have since demonstrated that the TGF- β signaling pathway suppresses glycolysis via inhibiting the mTOR pathway (97). In addition to smad3 signaling, one of the primary functions of TGF β in Treg differentiation may be to modulate the metabolism of activated T cells away from glycolysis. This may be mimicked by weak TCR stimulation in human T cells. The report that human Tregs have impaired differentiation when cultured with a glycolysis inhibitor therefore requires further assessment to delineate whether this is a true immunometabolic difference between human and mouse Tregs, or whether this observation is an artefact of the experimental design. This illustrates the necessity to confirm the basic immunological findings with human cells.

Delineating the optimal metabolic requirements for Treg differentiation and function is essential to guide the development of this exciting field. Ultimately, a precise definition of these requirements in human Tregs may open new experimental avenues and generate insights that can be utilised by those translating Tregs into clinical therapies.

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Figure 1. Overview of metabolic pathways in Tregs

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 Glycolysis: catabolic pathway of glucose. Glucose is catabolised into either lactate (aerobic glycolysis), acetyl-CoA entering TCA cycle or intermediates for other anabolic pathways including PPP. FAO: catabolic pathway of extracellular and intracellular fatty acids. FAS: anabolic pathway of fatty acids derived from cytosol Acetyl-CoA. Glutaminolysis: catabolic pathway of glutamine. Glutamine is catabolised into α -ketoglutarate fueling into TCA cycle.
 (ACC:Acetyl-CoA carboxylase, Akt:Serin/Threonin kinase, AMPK:Adenosine monophosphate-activated protein kinase,CPT1: Carityl-palmitate transferase 1, ETC:Electron transport chain, FAO:Fatty acid oxydation, FAS:Fatty acid synthesis, Foxo:Forkshead box O, Foxp3:Forkshead box P3, GCK:Glucokinase, HIF-1 α :Hypoxia-inducible factor 1 α , HK:Hexokinase, Lkb1:Liver kinase B1, mTORC:Mammalian target of rapamacin complex, OXPHOS:Oxidative phosphorylation, PI3K:Phosphoinositide 3-kinase, PPP:Pentose phosphate pathway, PTEN:Phosphatase and tensin homolog, TCA cycle: Tricarboxylic acid cycle)

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(ACC:Acetyl-CoA carboxylase, Akt:Serin/Threonin kinase, AMPK:Adenosine monophosphate-activated protein kinase,CPT1: Carityl-palmitate transferase 1, ETC:Electron transport chain, FAO:Fatty acid oxydation, FAS:Fatty acid synthesis, Foxo:Forkshead box O, Foxp3:Forkshead box P3, GCK:Glucokinase, HIF-1 α :Hypoxia-inducible factor 1 α , HK:Hexokinase, Lkb1:Liver kinase B1, mTORC:Mammalian target of rapamacin complex, OXPHOS:Oxidative phosphorylation, PI3K:Phosphoinositide 3-kinase, PPP:Pentose phosphate pathway, PTEN:Phosphatase and tensin homolog, TCA cycle: Tricarboxylic acid cycle)

Figure 2. Metabolic control of Treg differentiation

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Amino acids induce mTOR signalling which negatively regulate FoxP3 expression via inhibition of Foxo1/3 and antagonising TGF- β signalling. 2-HG derived from α -ketoglutarate and SCFA also epigenetically inhibit FoxP3 expression. Intracellular fatty acids via FAS and glycolysis inhibit Treg differentiation by an unknown mechanism.

(AMPK:Adenosine monophosphate-activated protein, CNS:Conserved non-coding sequence, FAO:Fatty acid oxydation, FAS:Fatty acid synthesis, Foxo:Forkshead box 0, Foxp3:Forkshead box P3, GCK:Glucokinase, HIF-1 α :Hypoxia-inducible factor 1 α , LCFA:Long-chain fatty acid, mTORC:Mammalian target of rapamacin complex, SCFA:Short-chain fatty acid, TCA cycle: Tricarboxylic acid cycle, TGF- β : Transforming growth factor-beta)

Figure 3. Metabolic control of Treg function

Figure 3. Metabolic control of Treg function

Metabolic requirements of Tregs are context-dependent. Activated Tregs have a high metabolic rate, requiring glycolysis that is induced by mTORs and HIF1 α in secondary lymphoid organs for clonal expansion and migration into peripheral tissues. However, these negatively regulate Treg suppressive function in the peripheral target tissues.

(Foxo:Forkshead box 0, Foxp3:Forkshead box P3, GCK:Glucokinase, HIF-1 α :Hypoxia-inducible factor 1 α , mTORC:Mammalian target of rapamycin complex, OXPHOS:Oxidative phosphorylation, PTEN:Phosphatase and tensin homolog)

Table 1 – Comparison of the importance of metabolic pathways. Adenosine monophosphate-activated protein kinase (AMPK); α -ketoglutarate (α -KG); essential amino acids (EAA); fatty acid oxidation (FAO); fatty acid synthesis (FAS); hypoxia-inducible factor 1 α (HIF-1 α); hypoxia-inducible factor 2 α (HIF-2 α); Regulatory T cells (Tregs).

	i/pTreg metabolism (Treg differentiation)	tTreg metabolism
Amino acids	EAA and glutamine deprivation enhance Treg differentiation via	EAA and glutamine are required to maintain metabolic fitness of activated

	<p>mTOR inhibition (29,30,38,39).</p> <p>α-KG derived from glutamine inhibits Treg differentiation by increasing TSDR methylation (40).</p>	Tregs (39,66).
Lipids	<p>(Long chain) FAO inhibition does not affect Treg differentiation (47).</p> <p>FAS inhibition enhances Treg differentiation (48,49)</p>	<p>Inhibition of cholesterol synthesis impairs Treg suppressive function (64,87).</p> <p>FAO is essential to maintain Treg mitochondrial homeostasis (89,90).</p>
Glycolysis	Glycolysis inhibition enhances Treg differentiation (55,58).	<p>Glycolysis impairs Treg suppressive function (69,70,79).</p> <p>Glucokinase expression is required for Treg migration (84).</p>
Hypoxia (+HIF signaling)	Context-dependent: Hypoxia enhances Treg differentiation, but HIF-1 α supports Th17 skewing by increasing glycolysis in the presence of IL-6 (55-59).	<p>HIF-1α and HIF-2α negatively regulate Treg suppressive function (72-75).</p> <p>HIF-1α is required for Treg migration (56,72).</p>