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Navigating T Cell Immunometabolism in Transplantation

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Abstract

Recently, a new discipline termed “immunometabolism” has transformed the field of immunology. It encompasses the study of the intrinsic metabolic pathways of different immune subsets and their impact on cellular fate and function. For instance, broadly speaking, pro-inflammatory cells depend upon glycolysis and glutamine oxidation while cells involved in anti-inflammatory response such as Foxp3⁺ regulatory T cells (Tregs) utilize predominantly fatty acid oxidation. However, while a useful paradigm, this actually is a reductionist view and the engagement of these metabolic pathways is not mutually exclusive between these subsets. Over the past several years, new insights and new methods to better dissect, define, and harness the metabolic properties of immune cells for immunotherapeutic purposes have come to the forefront. In this review, we will discuss the metabolic heterogeneity of different T cells subsets as well as basic principles of integrative technologies, such as metabolomics, that can be used to better understand the metabolic signatures of immune responses. Given the interest of exploiting this information in the context of transplantation, we will highlight the scope of immunometabolism in unraveling novel mechanisms of immune regulation that can be manipulated to promote Treg stability and function while inhibiting T effectors to establish long-term transplantation tolerance.

Keywords

Metabolomics; Regulatory T cells; immunometabolism; glycolysis; mitochondria

Introduction

In recent years, a new field called “Immunometabolism that is at the intersection of immunity and metabolism has emerged as the next big frontier in Immunology”¹. This field encompasses the study of the distinct metabolic programs in various immune cell subsets that influence their functionality and differentiation². Resting immune cells generate and utilize only minimal energy; ie the engine is on idle and their needs are modest. However, upon activation, immune cells become metabolically active and shift their metabolism from a catabolic state to an anabolic state, via aerobic glycolysis, to meet their bioenergetic demands. This is termed as “Warburg Effect”, a feature first identified by Dr. Otto Warburg

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in 1924 in cancer cells, where glucose derived pyruvate is shunted to lactate instead of being metabolized in the mitochondria despite the presence of abundant oxygen, hence the term “aerobic glycolysis”³(Fig. 1). Warburg metabolism is usually associated with a process called “anaplerosis” which is the oxidation of energy substrates such as glutamine to replenish tricarboxylic acid (TCA) cycle intermediates in the mitochondria (Fig. 1). Despite being less energy efficient, the shift towards aerobic glycolysis, away from mitochondrial metabolism, is beneficial for cells as it generates major glycolytic intermediates that are precursors to anabolic processes such as the synthesis of nucleotides, amino acids and lipids (through the pentose phosphate pathway, serine biosynthetic pathway, and the de novo fatty acid synthesis pathway, respectively) which are all required for cell growth and proliferation²(Fig. 1). In contrast to activated T effector cells and immunogenic/pro-inflammatory antigen-presenting cells, such as M1 macrophages and dendritic cells which rely upon the above mentioned metabolic programming, anti-inflammatory cells such as M2 macrophages, CD8 memory cells and differentiated regulatory T cells rely instead upon fatty acid oxidation (FAO) in the mitochondria²(Fig. 1). These metabolic programs occur generally due to alterations in PI3K/Akt/mTOR (the mammalian target of rapamycin) signaling axis. For instance, pro-inflammatory cells are typically associated with elevated signaling of this axis while anti-inflammatory cells are less dependent on it. Consequently, these signaling differences in turn give rise to specific metabolites that influence the functional differentiation of these cells. Together, these studies not only suggest how cellular processes are intimately linked to metabolism but also indicate that different types of immune cells may be differentially sensitive to various metabolic manipulations, owing to their specific metabolic signatures^{4, 5, 6}.

Among the several immune cell types that promote tolerance, Foxp3+ Tregs have particularly been under the spotlight, as they constitute one of the main mechanisms of immune regulation. Given the critical role of Tregs in immune regulation, in this review we will focus on comparing their metabolism to T effector cells while providing an overview of various new strategies that one can employ to study this phenomena in the context of experimental and preclinical transplantation studies.

Immunometabolism: The new kid on the block

Immune cells engage in a bidirectional interaction between extrinsic cues and intrinsic metabolic signaling. The role of how metabolic pathways control different aspects of lymphocyte biology has been summarized previously². Here we highlight some of the most recent developments that show how metabolic programming ultimately affects an immune cell's activation status and its functional differentiation.

T effector cells

As activated T cells transition from a quiescent state to an activated state, they increase their biomass required for cell growth and proliferation. During this process T cells have been shown to undergo asymmetric cell division when each of the daughter cells differentiates either towards an effector or memory phenotype cell. These fate decision processes are mediated by the metabolic programming driven by the asymmetric distribution of the

activity of mammalian target of rapamycin complex 1 (mTORC1) that correlates with c-Myc distribution ^{7, 8}. For example, c-Myc^{hi} daughter cells show increased mTORC1 activity and upregulate Glut1, a key surface membrane transporter of glucose into the cell, and increase their glycolytic activity, which promotes effector cell function. Conversely, the cells with lower mTORC1 activity utilize lipid metabolism, which promotes the generation of long-lived memory cells ⁶. The increase in glycolysis in cells of effector lineage, is not only required to meet the metabolic demands of cell growth but is also required for optimal production of pro-inflammatory cytokines such as IL-2 and IFN- γ ⁹. This was shown to be dependent upon the sequestration of bifunctional glycolytic enzymes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that normally binds to the 3' untranslated region of IFN γ and inhibits its translation ⁹. Interestingly, another bifunctional glycolytic enzyme, lactate dehydrogenase (LDH α), has also been shown to play a role in promoting IFN- γ expression via epigenetic modifications (via maintenance of high level of acetyl CoA and augmenting histone acetylation) ¹⁰. These studies suggest that metabolic control of T effector function can be multifactorial. Consistent with these findings, genetic deletion or pharmacological inhibition of Glut1 disrupts optimal function of alloreactive effector T cells and ameliorates graft-versus-host disease (GVHD) mortality in mice undergoing bone marrow transplantation ^{6, 11, 12}. In a stringent skin transplantation model, inhibition of both glucose metabolism and glutamine metabolism (considered as an anaplerotic source of nucleotides and polyamines biosynthesis during T cell proliferation) by drug-combination therapy prevented acute rejection ¹³. These studies therefore suggest that metabolism is intimately linked to the immune cellular processes and their effector function.

T regulatory cells

The importance of glycolytic metabolism for T effector cells is further highlighted in studies that show that inhibition of the glycolytic-lipogenic pathway during cell culture prevents Th17 cell differentiation, and instead promotes the generation of in vitro induced Tregs (iTregs) ^{14, 15}. Glutamine deprivation of naïve CD4⁺ T cells, another critical fuel source of T effector cells, also leads to differentiation into iTregs even under Th1 effector cell type skewing conditions ¹⁶. These cell fate decisions of T cells are dictated by differential mTORC1 signaling where higher mTOR activity is critical for effector cell differentiation while lower mTOR activity favors iTreg generation. These cells instead depend upon AMP-activated protein kinase (AMPK) signaling, a reciprocal sensor to mTOR, that facilitates mitochondrial FAO which is required for the induction of Foxp3. Thus, blockade of mTOR with rapamycin or activation of AMPK via metformin, an anti-diabetic drug, increases iTreg lineage development and their suppressive function ⁴.

The in vivo counterpart of iTregs cells are termed peripherally-derived Tregs (pTregs). In accordance with published guidelines, we use “iTreg” only for in vitro generated Tregs and “pTreg” for the in vivo generated population¹⁷. Both these cells originate from naïve T cells and acquire Foxp3 following peripheral stimulation by antigen under noninflammatory conditions, eg, in the presence of TGF- β (Fig. 2). The maintenance of Foxp3 expression postactivation in both iTregs and pTregs is associated with the partial demethylation of CpG motifs in the Treg specific demethylation region (TSDR) within the *Foxp3* gene. However, both these cell types are believed to have inherent lineage instability, as they fail to fully

demethylate these CpG motifs and thus may lose Foxp3 expression and acquire effector function especially in conditions of inflammation^{18, 19}. In contrast to iTregs, thymically derived Tregs (tTregs) maintain full demethylation of the Foxp3 TSDR, and are considered more functionally stable²⁰

It is becoming increasingly evident that the metabolic requirements of tTregs may be different than iTregs. For instance, mTORC1 dependent glycolytic-lipogenesis that promotes cholesterol biosynthesis is indispensable for functional fitness of tTregs. These metabolic processes are associated with the upregulation of Treg signature markers such as CTLA-4 and ICOS and their suppressive function²¹. Furthermore, it was reported recently that freshly isolated human ex vivo Tregs are highly glycolytic and engage in both glycolysis and FAO when cultured in vitro²². These findings highlight the importance of mTORC1 signaling and glycolytic metabolism in tTreg stability and function in contrast to AMPK signaling and FAO for iTregs. However, there have been reports that suggest otherwise. For instance, a recent study showed that although TLR-induced mTORC1 signaling promotes tTreg proliferation through enhancing glycolysis and Glut1 expression, it simultaneously impairs tTregs suppressive capacity²³. Autophagy deficiency can also enhance c-Myc function and mTORC1 signaling resulting in increased glycolytic metabolism that is associated with defective tTreg stability and function²⁴. Furthermore, Treg-specific deletion of PTEN, a negative regulator of PI(3)K, enhances a glycolytic program that is associated with compromised function and lineage stability of tTregs²⁵. Finally, studies in human iTregs (induced by suboptimal stimulation without IL-2) have recently shown that they can in fact depend upon glycolysis to sequester enolase1, a bifunctional glycolytic enzyme that represses the E2 variant of Foxp3 required for their suppressive function. These studies show that while gaining the ability to undergo enhanced glycolysis may be detrimental to tTreg stability and function, it doesn't necessarily equate towards loss of suppressive function in iTregs²⁶. Together these contradictory findings suggest that tTregs and iTregs (pTregs) have complex but clearly different metabolic requirements in different contexts.

The discrepancies in these studies may be attributed in part to species (mice vs human) differences or variations in experimental systems. However another possibility should be considered as well. The population of freshly isolated Foxp3+ Tregs from secondary lymphoid tissues is likely comprised of both tTregs and pTregs. Hence the metabolic differences seen in these studies may just be a reflection of heterogeneity within Treg population. At present, there is yet no reliable way to distinguish the 2 subsets of Tregs. Recently 2 markers, neuropilin 1 (Nrp1), a semaphorin III receptor, as well as the transcription factor Helios have been reported to be expressed by exclusively by tTregs^{27,28}. However, unlike mouse Tregs, human Foxp3+ T cells do not express Nrp1^{29, 30, 31}. In addition, Helios can be induced upon T cell activation and hence cannot be used definitively to distinguish tTregs and pTregs³². Therefore, a lack of good distinguishing markers makes it difficult to study these 2 populations. Despite these limitations, studies showing biological differences between tTregs and pTregs in terms of their TCR repertoire owing to their origin, demethylation of TSDR region of the Foxp3 promoter (indicated above) have been reported³³. Figure 2 summarizes the key distinct features between these 2 subsets that may in turn be linked to their differing metabolic signatures.

Strategies to evaluate immunometabolism

Understanding the key mechanisms and metabolic functions of immune cells, like that of other cell types, can be broadly classified into 2 levels (1) basic metabolic characterization, and (2) advanced metabolite profiling. Several new analytical technologies have recently emerged that can provide a holistic picture of the metabolic signatures of immune cells under physiological and pathological conditions. Here, we will summarize the basic aspects of these technologies that serve as complementary tools along with traditional metabolic assessment strategies for the study of immunometabolism.

Basic Metabolic Characterization

The rapid and a reliable assessment of cellular bioenergetics in real time using the Seahorse XF Bioanalyzer has increasingly become a useful technique to profile the overall picture of the metabolic function of cells. The measurement of oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) serve as surrogate markers for mitochondrial metabolism and glycolysis, respectively. The measurement of OCR after the sequential addition of inhibitors such as oligomycin (mitochondrial ATP synthase inhibitor), trifluoromethoxy carbonyl cyanide phenylhydrazine (FCCP, proton ion hole uncoupler) and complex 1 and 3 inhibitors such as rotenone and antimycin help gauge one of key parameters called the spare respiratory capacity. This is a unique feature shared between memory and regulatory T cells that rely heavily upon FAO⁴. In contrast to this, measurement of ECAR after addition of glucose, oligomycin and 2DG helps in the assessment of cell's glycolytic capacity, another key feature that is synonymous with T effector cell metabolism and activated bone marrow cells^{14, 34}. Along with parallel analyses of metabolic signaling pathways such as mTOR and AMPK signaling, the expression of nutrient transporters involved in glucose, glutamine and FA uptake and measuring the expression of key rate limiting enzymes and their activities via traditional methods of qPCR, immunoblotting and flow-based methods can help to lay the foundation for understanding the basic metabolic phenotype of immune cells.

Along these lines, assessing mitochondrial physiology is yet another powerful tool for understanding the metabolic phenotype of immune cells. In this regard, mitochondria distinctly play a fundamental role as bioenergetic hubs for mitochondrial oxidative phosphorylation via TCA cycle and electron transport chain to generate ATP. Mitochondrial dynamics have been shown to be crucial in dictating T cell fate between naïve, effector and memory T cell states. Recently, the morphological adaptation of mitochondria in memory cells towards a fused state as opposed to a fissioned state (effector cells), was shown to be a critical for T cell longevity, a key attractive feature required for cells to survive in vivo³⁵. Furthermore, assessing mitochondrial function via flow-based assessments for mitochondrial mass, membrane potential and reactive oxygen species (ROS) production aids in the understanding of the cellular bioenergetics that is linked to stress response. iTregs engaging in FAO have been shown to be equipped with higher levels of ROS, and treatment with ROS scavengers decreases Foxp3 expression suggesting the critical role for ROS in serving as a metabolic signaling molecule for Treg function¹⁴.

Advanced Metabolomic profiling

While Seahorse XF technology is a good tool to obtain a quick snapshot of immune cell metabolic activity (ECAR and OCR changes), it offers only a limited picture as it doesn't indicate which specific pathways are being utilized in cells and to what extent.

Metabolomics offers a solution as it delves deeper into the comprehensive measurement of metabolites and their usage in various pathways. Specifically, this technology comprises of the identification and quantification of thousands of metabolites usually under the molecular weight of 1200 kDa. These small molecule metabolites include sugars, amino acids, fatty acids, polyamine, antioxidants and the many other classes of compounds. Metabolomics is similar to other -omic approaches ie genomics for genes, transcriptomics for RNA and proteomics for proteins, and serves as integrative complementary tool that allows not only for the identification of the new biomarkers but also mechanistic insights into key metabolic pathways and their checkpoints (Fig. 3A). Two analytical platforms, namely nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) coupled usually with liquid chromatography (LC) to separate individual metabolites are the most commonly used in metabolomic analyses ³⁶. Despite the potential impact of several fluctuating environmental and intrinsic factors on cellular metabolism, integrative metabolomic data analyses (with other -omic studies or biostatistical models) can discriminate stable metabolic changes from individual variations and help construct meaningful metabolic networks ³⁷. In order to obtain a comprehensive picture of the metabolic profile, 2 major metabolomic approaches have been currently employed. They comprise of (A) determining the abundance of the metabolites at steady state levels and (B) understanding the directionality and the rate of the metabolic flow ³⁸ (Fig. 3B).

(A). Abundance—Untargeted metabolomic approaches that profile the levels of several metabolites are currently being integrated to an already established genomic/transcriptomic analyses platforms using biofluids such as blood, plasma and urine samples in the aim to identify new biomarkers in kidney, liver and heart transplantation and has been reviewed elsewhere ³⁹. Similarly, such approaches have been increasingly adopted to better assess the abundance of metabolites in key metabolic pathways in immune cells under various conditions. One of the first examples in lymphocytes, integrating metabolomics with traditional fuel flux measurements that use radiolabeled carbon substrates, used an integrative approach to assess the steady state levels/concentrations of key metabolites in glycolysis, glutamine and FAO post activation. Consistent with their metabolic flux findings, this study validated that T cell activation caused a dramatic increase in the accumulation of metabolites involved in glycolysis and glutamine metabolism while causing a concomitant decrease in FAO, all of which was shown to be driven by transcription factor c-Myc ⁴⁰. Since then, many studies adopting this strategy have helped gain insight into the metabolic programming during T cell activation. For instance, metabolomic profiling assays assessing donor T cells that induce GVHD also revealed the increase in metabolites involved in glycolysis and glutamine metabolism and decrease in FAO that have been shown to be unexpectedly dependent upon the expression of PDL1 on T cells ^{11, 41}. Although this form of profiling approach has many advantages in terms of its ability to map and quantify metabolic pathways, these steady-state analyses cannot determine whether the change in the quantity of certain metabolites are due to the changed activity of a synthesizing enzyme, or a

consuming enzyme. Follow up of abundance studies with stable isotope tracer analysis is therefore the next step in fully evaluating the metabolic signature of cells.

(B). Directionality and Metabolic flux—Stable isotope tracer analysis helps in assessing the metabolic directionality and the flux and hence results in a better understanding of the mechanistic dynamics of the metabolic pathways that are involved. Traditionally, stable isotopes used to study metabolic process constitute labeled carbon (^{13}C), hydrogen (^2H), and nitrogen (^{15}N). These are used both in vitro and in vivo to determine the enrichment of carbon labeling in metabolites over time. Each of the labeled species of a metabolite is term as an “isotopomer”. Techniques of LC/MS or NMR obtain isotope fraction labeling and mass isotopomer distribution information. This determines the relative abundance of labeled carbon species and the specific positions of carbon labeling in metabolite pools. Together, this data can help reveal the metabolic fate of substrates. Fig. 3C shows a simplified example of a roadmap to decipher the ^{13}C labeling patterns in citrate using uniformly labeled ^{13}C glucose. The incorporation of labeled carbon into various isotopomers of a metabolite [in this case citrate] gives rise to “mass isotopomer distribution” for citrate. By evaluating the total ^{13}C labeled enrichment fraction as well as examining the mass isotopomer distribution, one can interpret relative directionality and flux of metabolic pathways used under steady state and over time. Using such studies, the importance of glutamine metabolism in activated T cells has been recently highlighted using ^{13}C labeling of 3 primary substrates under different culture conditions. Studies using uniformly labeled U- ^{13}C labeled glucose during glutamine starvation of activated T cells indicated a depletion of TCA cycle intermediates, such as citrate and fumarate (via the assessment of reduction of relative abundance of ^{13}C labeled citrate or fumarate) that was not compensated by glucose, indicating the absolute requirement of glutamine in serving as an anaplerotic source for TCA cycle intermediates during T cell activation ⁴². In addition, under glucose limiting conditions, glutamine can compensate as an energy source to generate glycolytic intermediates such as pyruvate (increased in the relative abundance of ^{13}C label in pyruvate) for its use in TCA cycle via the process of glutamine decarboxylation, a process shown to be dependent upon AMPK signaling ⁴². Interestingly other ^{13}C labeled glutamine studies, also showed higher ^{13}C incorporation from labeled glutamine in ribose rather than from the traditional source of labeled glucose in activated donor T cells during GVHD suggesting the role of glutamine metabolism in feeding biosynthetic pathways such nucleotide biosynthesis ³⁴. Furthermore, studies showing the distribution of labeled palmitate, another fuel source in different glutamate isotopomers suggested that palmitate can also serve as another source for glutamine intermediates required for donor T cell activation during GVHD. Together these stable isotope-labeling studies underscore the precise importance of glutamine and palmitate metabolism in serving as anaplerotic energy sources for biosynthetic processes such as nucleotide synthesis and mitochondrial metabolism during T cell activation.

Interestingly, ^{13}C incorporation from labeled glucose to palmitate was minimal in donor T cells during GVHD indicating that contribution to FA synthesis from the glycolytic lipogenesis is very little in these cells ³⁴. In contrast to these studies, ^{13}C incorporation from U- $^{13}\text{C}_6$ labeled glucose to palmitate has been shown to be substantial in T cells that have been differentiated towards the Th17 cells but not iTregs in conditions of autoimmunity ¹⁵.

Together, these studies indicate the advantage of labeling studies in dissecting the precise metabolic signatures of immune cells in different scenarios. These studies bring in another layer of understanding that suggest that metabolic wiring of immune cells is plastic owing to the context of immune cell activation.

In addition to determining the directionality, stable isotope labeling analyses can be combined with computational models to provide the velocities of biochemical reactions in key metabolic nodes under physiological and pathological states. This aspect is beyond the scope of this review and is covered elsewhere³⁷³⁶. Together, these studies highlight the power of these analyses to decipher the unique metabolic dependencies and flexibilities in immune cells that might be affected either due to intrinsic factors (specific fuel choices made during cell differentiation) or extrinsic factors such as substrate limitations and can be used to determine these differences perhaps during metabolic manipulation.

Potential Applications of immunometabolism in Transplantation

Since metabolic alterations represents a state of immediate cellular responses to stresses, metabolomics measurements have been suggested as a strategy for rapid assessment of graft function and rejection. For instance, ROS and metabolites such as tri-methylamine N-oxide (TMAO) are typically produced during ischemia/reperfusion injury³⁹. Further, metabolic profiling studies of serum after kidney transplantation revealed that the intermediate of tryptophan metabolism, kynurenine, is altered during acute rejection³⁹. Now, with the emergence of the field of immunometabolism and the advancement of metabolomic techniques, increasing attention is being paid to using these approaches not only to identify novel drug targets, but also to define key metabolic nodes that can serve as prognostic biomarkers to monitor recipient's immune status during transplantation (Fig. 4). Given the emergence of Treg cellular therapy for tolerance induction and maintenance, metabolic strategies to generate functionally stable and long-lived efficacious Treg cells either during exvivo T reg cell expansion protocols (described below) are an attractive option and could enable the use of drugs which are not suitable for in vivo administration.

Immunometabolic Intervention

The evidence of the feasibility of metabolic manipulation for immune mediated conditions began emerging first in models of allergy and autoimmune diseases. For instance, administration of the AMPK activator metformin, which blocks lipogenesis and activates FAO, increased the frequency and number of Tregs in a mouse model of asthma⁴. Additionally, experimental use of another AMPK activator, AICAR, has been shown to suppress IFN- γ production by effector T cells⁴². Blocking the glycolytic-lipogenic pathway in experimental autoimmune encephalitis (EAE) using inhibitors such as 2DG (which blocks glycolysis by inhibiting hexokinase (HK)) and soraphen (which inhibits glycolytic lipogenesis by blocking acetyl-coA carboxylase (ACC)) (Fig. 1), decreased Th17 cell while reciprocally promoting Tregs¹⁵. Furthermore, the dual blockade of glycolysis by 2DG and mitochondrial metabolism by metformin normalized the overall metabolism of CD4+ T cells and decreased symptoms in murine model of SLE⁴⁴.

Interestingly, similar strategies in transplantation models have yielded promising results. For instance, a combination therapy of 2DG+ (inhibition of glutamine metabolism via DON+ metformin delayed or prevented rejection in fully mismatched skin and heart transplant experiments in mice ¹³. This was accompanied by the increase in the proportion of Tregs suggesting that balance between Tregs and T effectors had been modified by metabolic intervention. Furthermore, in a model of GVHD, the small molecules BZ423 which blocks mitochondrial respiration, or etomoxir which blocks FAO (Fig. 1), ameliorate the symptoms of GVHD by blocking donor reactive T cells while sparing bone marrow cells that are predominantly glycolytic. Interestingly, glycolysis blockade using PKF15 (which blocks the key glycolytic enzyme PFKFB3 also blocked donor reactive T cells and ameliorated the symptoms in a different model of GVHD (Fig. 1) ¹¹. These studies suggest that activated T cells may be capable of preferentially engaging in 1 metabolic program over the other in a context dependent manner. Alternatively, the metabolic demands of a rapidly proliferating population of cells, such as in GVHD, requires optimal usage of all metabolic pathways. Interestingly, freshly isolated human Tregs can engage in both glycolysis and FAO²². As described earlier, glycolysis is required for optimal Foxp3 induction in human iTregs, suggesting that strategies that can enhance glycolysis in these cells may be useful ²⁶. In this context, UK5099, an inhibitor of mitochondrial pyruvate carrier (MPC) (Fig. 1 and Fig. 5), which was recently shown to impair pyruvate metabolism and enhance aerobic glycolysis resulting in metabolic reprogramming of cancerous cells towards stem-like cells, could serve as a potential approach to boost glycolysis and enhance Foxp3 expression in human iTregs ^{45, 46}.

Given the exponential rise of recent immunometabolic studies that are beginning to reveal the impact of different metabolic pathways on immune function, there is tremendous opportunity for the development of potentially new metabolic modulators ^{2, 47}. These modulators can be broadly classified into 4 categories, ie; 1) Targeting signaling pathways 2) Targeting metabolic enzymes 3) Targeting mitochondrial reactions 4) Targeting epigenetic alterations. They have been summarized in Fig 5. Given the distinct metabolic requirements of different subsets of immune cells, the idea of cellular selectivity with minimal toxicity is truly highlighted by the promise of metabolic manipulation in organ transplantation.

Integration of metabolomics with other “omics

In addition to providing novel mechanistic insights into immune cell function that can be modulated, immunometabolism is starting to usher in a new wave of mechanistic studies and biomarker discovery that can be used during immune monitoring post transplantation. The use of metabolomics as a strategy to determine biomarkers to monitor graft function has already been shown in models of kidney ischemia injury, liver and heart transplantation ⁴⁸. Despite posing complex challenges, recent work showing the integration of metabolomics with T cell proteomics and phosphoproteomic approaches indicates a potential for establishing a multi-marker monitoring strategy for immune cells during transplantation. For instance, a recent study adopting this multi-level omics approach revealed the critical role for mitochondrial enzyme cox10 in facilitating T cell quiescence exit and T cell differentiation indicating the importance of remodeling of mitochondrial bioenergetics during T cell activation ⁴⁹. Analysis of the proteomic landscape of activated human Tregs

also revealed enrichment of proteins and enzymes involved in mitochondrial metabolism suggesting that proteins in mitochondrial pathway represents a common functional module linked to T cell activation in both T effectors and Tregs ²².

At the same time, the next realm that is beginning to emerge is the influence of metabolism on events such as transcriptional regulation, chromatin modeling and epigenetics that are linked cell fate and functionality. For instance, proteomic analyses have revealed the upregulation key transcriptional regulators that regulate T cell survival in response to amino acid arginine levels ⁵⁰. As described earlier, increased glycolysis due to LDH α activity can affect histone acetylation of IFNG loci and regulate its expression in T effector cells ¹⁰. Likewise, gene regulatory processes have been shown to dictate their stability and function in Tregs. Recently, metabolites such as vitamin A have been shown to increase histone acetylation of Foxp3 gene promoter while also facilitating CpG demethylation of Foxp3 locus, both of which are required for optimal expression of Foxp3 ⁵¹. Similarly, metabolites such as vitamin C have recently been shown to increase the activity of TET enzymes that are crucial for DNA demethylation of Foxp3 locus and stability of Foxp3 expression similar to glutamine metabolism intermediate α -ketoglutarate (aKG) that also activates TET activity and histone modifications in hematopoietic stem cells ⁵²(Fig. 5). Furthermore, newer studies indicate the importance of chromatin modifiers EZH2, (a protein shown to be repressed in response to glucose restriction), in regulating the expression of several Treg lineage genes required for the establishment of their Treg identity ⁵³. Hence integration of metabolomics studies with other approaches such as epigenomics can help obtain a comprehensive picture of the various metabolic functional modules and the differentially regulated molecular circuits in immune cells especially in the context of metabolic manipulation.

Metabolic manipulation in Tolerogenic cell therapy: A brief perspective

The safety and potential therapeutic efficacy of Treg cell therapy has been reported in Phase I/II trials of BMT and type I diabetes, and currently being conducting for solid organ transplantation. Efficient ex vivo expansion of a stable and functional Treg population is a prerequisite for their successful implementation in immunotherapy and novel ways to promote high quality Tregs are currently being explored. Given the intrinsic role of immunometabism in regulating various aspects Treg biology and function, broadening the application of metabolic manipulation to Treg expansion protocols hold tremendous promise. Recent reports using a culturing strategy with rapamycin show encouraging signs of enhanced cell expansion and Treg stability ⁵⁴. In addition to mTOR inhibitors, existing and emerging metabolic modulating targets that may have selective desired effects for promoting Treg proliferation, stability and function are being currently considered for this purpose.

In addition to polyclonal or donor antigen specific Treg therapy, recent advancements in chimeric antigen receptors (CARs) technology and its effects on metabolic properties of cells are beginning to provide new insight for metabolic manipulation for Treg cell therapy. In the field of cancer, CAR T cells with intracellular 4-1BB signaling domain has been recently reported to maximize antitumor activity by favoring oxidative metabolism and

promoting central memory differentiation⁵⁵. The modular nature of the CAR, extracellular antigen-binding domain fused to intracellular cell signaling domains, allow for optimization by replacement of various components. Given the role of PD1 and Ctla4 in initiating metabolic pathways in blocking glycolysis while enhancing FAO, both serve as attractive candidates for Treg therapy including CAR Treg therapies to induce FAO and reduce glycolysis in Tregs, a process essential for the suppressive function. This indicates an indirect strategy to modulate metabolism and possibility to selectively manipulate metabolic signaling pathways in cell type specific manner.

Conclusions

The field of immunometabolism until recently has been primarily focused in models of infectious disease or in cancer immunology. The objective of this review was to provide an overview of this field and an insight into various strategies that can be used to navigate it from a tolerance standpoint. Given the rise of CAR and CRISPR engineering technologies, and the wide ranging effects of metabolic changes on epigenetic regulation and cellular fate and function, the goal to shape a personalized immune response with favorable balance of T effectors/Tregs in conditions of autoimmunity and transplantation may no longer seem that far-fetched.

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Abbreviation

Treg	regulatory T cell
TCA	tricarboxylic acid
FAO	fatty acid oxidation
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
LDH	lactate dehydrogenase
GVHD	graft-versus-host disease
iTreg	in vitro induced Treg
AMPK	AMP-activated protein kinase
pTreg	peripheral derived Treg
tTreg	thymically derived Treg
TSDR	Treg specific demethylation region

Nrp 1	neuropilin 1
OCR	oxygen consumption rate
ECAR	extracellular acidification rate
FCCP	trifluoromethoxy carbonyl cyanide phenylhydrazone
ROS	reactive oxygen species
NMR	nuclear magnetic resonance spectroscopy
MS	mass spectrometry
LC	liquid chromatography
TMAO	tri-methylamine N-oxide
EAE	experimental autoimmune encephalitis
HK	hexokinase
ACC	acetyl-coA carboxylase
MPC	mitochondrial pyruvate carrier
aKG	alpha-ketoglutarate
CAR	chimeric antigen receptor

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Fig 1. Roadmap of key metabolic pathways

This figure represents the utilization of 3 major fuel substrates, glucose, fatty acids and amino acids (glutamine) via 3 key metabolic pathways namely glycolysis (conversion of glucose to pyruvate), fatty acid oxidation and glutaminolysis respectively. Glycolysis derived pyruvate has 2 fates. It can either be converted to lactate (defined as aerobic glycolysis), which is a feature of activated T cells and cancer cells, or it can be converted to acetyl coA, which is metabolized in the TCA cycle via glucose oxidation. In addition to glucose metabolism, fatty acids and glutamine serve as anaplerotic fuel sources that can be utilized in the mitochondrial TCA cycle as depicted. The electrons donated by the TCA cycle are associated with the generation of ATP in the electron transport chain and this process is defined as mitochondrial oxidative phosphorylation. The intermediates of all these metabolic pathways further feed into several biosynthetic pathways such as pentose phosphate and serine biosynthesis for nucleotide generation, protein synthesis and lipid biosynthesis, all of which are required for biomass accumulation. The key rate limiting enzymes that serve as targets for metabolic manipulation of these various metabolic pathways are highlighted in (red) while their respective inhibitors are highlighted in yellow. HK, hexokinase; 2DG, 2 deoxy glucose; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; PFK15, 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; MPC mitochondrial pyruvate carrier; PDH, pyruvate dehydrogenase; PDHK, pyruvate dehydrogenase kinase; DCA, dichloroacetate; CPT1, carnitine palmitoyltransferase I; ACC1, acetyl coA Carboxylase 1; SorA, sorafenA; aKG, alpha ketoglutarate; GLS, glutaminase; DON, 6-diazo-5-oxo-L-norleucine

Fig 2. Distinctions between tTregs and pTreg subsets

Tregs are broadly classified into 2 distinct populations. Thymic derived (tTregs) constitutively express Foxp3 and are believed to be selected by self-antigen stimulation during thymic selection. In contrast, peripheral derived Tregs (pTregs) are derived from peripheral naïve T cells in the presence of TGFβ and are found in peripheral tissues such as the gut. Naïve T cells activated in the presence of TGFβ+ IL-2 and are referred to as in vitro induced Tregs (iTregs).

Fig 3. Currently available strategies to estimate immunometabolism

(A) A schema of how metabolomics integrates with various levels of multi-platform omics approaches currently employed. (B) Components of metabolomics studies. (C) A schematic example of ^{13}C isotope label channeling into various glycolytic and TCA cycle metabolites from either uniformly labeled glucose. ^{12}C represents each of unlabeled carbon (clear circles) and M denotes the mass of a particular metabolite. The number assigned next to the mass, for instance M0 of glutamine indicates the mass of the unlabeled glutamine while M6 of glucose denotes the mass of 6 stable isotope ^{13}C labeled carbons of glucose (red circles). The figure shows an example of the labeling pattern in 3 isotopomers of citrate that have been derived from uniformly labeled U- $^{13}\text{C}_6$ glucose [M6] via various routes. Firstly, the reaction of the labeled acetyl CoA [M2] with unlabeled oxaloacetate (OAA) [M0] derived from unlabeled glutamine during the first round of the TCA cycle results in the generation of M2 species of citrate. Secondly, as this M2 species of citrate enters the second round of the TCA cycle, it results in the formation of M2 species of OAA that in turn reacts with labeled acetyl CoA again leading to the formation of M4 species of citrate. These 2 species indicate the generation of citrate via the normal TCA cycle route. Finally, if the pyruvate [M3] undergoes conversion to OAA via the reaction catalyzed by pyruvate carboxylase, it will generate M3 species of OAA. Its reaction with labeled acetyl CoA in turn will ultimately result in the generation of M5 species of citrate. Therefore the measurement of the total % of ^{13}C fraction enrichment in a particular metabolite and the mass isotopomer distribution

within that fraction can be plotted in terms of its relative abundance (as displayed above). Hence, stable isotope labeling studies can help resolve the nature of the metabolic activity.

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Fig 4. Potential Application of Immunometabolism in Transplantation

Schematic picture shows the potential application of immunometabolic techniques during the pre- and post-transplant stages. Patients receiving bone marrow transplantation are usually subject to treatment for nonspecific T cell depletion with chemotherapy, whole body irradiation, anti-thymocyte globulin (ATG), or their combination. Metabolic preconditioning to selectively suppress effector T cells while sparing Tregs would be beneficial. Furthermore, given the metabolic requirements of donor T cells during GVHD, incorporation of metabolic conditioning to inhibit effector T cells that can cause GVHD and induce more Tregs in vivo can serve as another potential intervention strategy. Additionally, Tregs may be adoptively infused into the patient to promote tolerance in these circumstances. This forms the basis for the ex vivo culturing of Tregs that can either use polyclonal, antigen- specific (generated by the expansion of host Tregs in response to donor APCs) or CAR Tregs. Metabolic manipulations of these populations during the activation or expansion phase can be used to generate Tregs that are both qualitatively and quantitatively effective to survive and be functional in vivo. Finally, immunometabolic assessment/ monitoring has the potential to serve as a non-invasive complementary tool to traditional assays in the risk management during acute and chronic rejection. Furthermore, these assays can also help pave way to the identification of newer biomarkers.

Fig 5. Potential Immunometabolic Intervention strategies

This figure summarizes the different metabolic inhibitors currently being investigated for their potential use in autoimmunity and transplantation. The intervention strategies are categorized into 4 groups based on their site of action. These include 1) signaling pathways 2) metabolic enzymes 3) Mitochondrial function 4) Epigenetic alterations. AMPK, AMP kinase; AICAR, 5-Aminoimidazole-4-carboxamide ribonucleotide; mTORC1, mammalian target of rapamycin complex 1; 2DG, 2 deoxy glucose; HK, hexokinase; CPT1, carnitine palmitoyltransferase I; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; PFK15, 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one; DCA, dichloroacetate; PDHK1, pyruvate dehydrogenase kinase 1; DON, 6-diazo-5-oxo-L-norleucine; GLS, glutaminase; SorA, soraphen A; ACC1, acetyl Co.A Carboxylase 1; MPC, mitochondrial pyruvate carrier; FA, fatty acid; FAO, fatty acid oxidation; aKG, alpha ketoglutarate; TET, ten-eleven translocation enzyme.