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Targeting Immuno-metabolism to Improve Anti-Cancer Therapies

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Abstract

The immunology community has made significant strides in recent years in using the immune system to target and eliminate cancer. Therapies such as hematopoietic stem cell transplantation (HSCT) are the standard of care treatment for several malignancies, while therapies incorporating chimeric antigen receptor (CAR) T cells or checkpoint molecule blockade have been revolutionary. However, these approaches are not optimal for all cancers and in some cases, have failed outright. The greatest obstacle to making these therapies more effective may be rooted in one of the most basic concepts of cell biology, metabolism. Research over the last decade has revealed that T cell proliferation and differentiation is intimately linked to robust changes in metabolic activity, delineation of which may provide ways to manipulate the immuno-oncologic responses to our advantage. Here, we provide a basic overview of T cell metabolism, discuss what is known about metabolic regulation of T cells during allogeneic HSCT, point to evidence on the importance of T cell metabolism during CAR T cell and solid tumor therapies, and speculate about the role for compounds that might have dual-action on both immune cells and tumor cells simultaneously.

Keywords

Adoptive cell transfer; Chimeric antigen receptor; Graft versus host disease; Hematopoietic stem cell transplant; Metabolism; Tumor infiltrating lymphocyte

Introduction

Many high-quality reviews on T cell metabolism have been published recently and the reader is encouraged to seek these out [1, 2]. In the classic paradigm, quiescent T cells rely on oxidative metabolism, where nutrients are transported into the mitochondria, fats are oxidized through fatty acid oxidation (FAO), intermediates are shunted into the Tricarboxylic acid cycle (TCA) to generate FADH₂ and NADH, and these reducing agents

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are used by the electron transport chain to generate ATP through oxidative phosphorylation (OXPHOS). When T cells become activated, signaling through the TCR and the increased metabolic demands of a rapid division, drive an increase in the rate of glycolysis, with active conversion of pyruvate to lactate instead of being shunted into the TCA cycle [3, 4]. This increase in glycolysis, despite the presence of sufficient oxygen, is often referred to as the “Warburg effect”, eponymously named for Otto Warburg’s seminal discovery of a similar aerobic glycolysis described in cancer cells at the beginning of the last century [5]. During resolution of an immune response, surviving T cells convert to memory T cells and again become reliant on oxidative metabolism [6–9].

Until recently, little was known about the process of lipid transport and lipolysis in T cells. That is beginning to change and the first of the new studies suggests that lipids can be generated *de-novo* inside of cells, rather than be transported from outside, followed by breakdown of lipid by intracellular lipases including lysosomal acid lipase (LAL) [10]. More recently, this viewpoint has expanded to demonstrate that both lipid uptake and synthesis are important for robust T cell proliferation following antigen recognition. Specifically, the mTORC1-PPAR γ pathway was found to be critical to drive fatty acid uptake in activated CD4⁺ T cells and this adaptation was absolutely necessary to achieve complete activation and rapid proliferation of both naive and memory CD4⁺ cells [11]. In addition, uptake of free fatty acids (FFAs) by fatty acid binding protein 4 and 5 (FABP4/FABP5) was determined to be critical for optimal performance of tissue resident memory T cells, and genetic knockdown of these vital proteins yielded T cells with poor protection against viral skin infections [12].

To generate energy from fat oxidation, cytosolic FFAs are conjugated to an acyl group by coenzyme A, chaperoned to the mitochondria, and the CoA moiety is replaced with carnitine by the molecule carnitine palmitoyl transferase 1 alpha (CPT1 α). This acyl-carnitine species is then transported across the mitochondrial membrane by carnitine translocase, followed by deconjugation of carnitine by CPT2, which converts acylcarnitines back to a long-chain acyl-CoA molecules. Intramitochondrial Acyl-CoA moieties then become available for catabolism through the process of β -oxidation [13]. The end-product of FAO is Acetyl-CoA, which when shuttled into the TCA cycle, produces the reducing equivalents NADH and FADH₂ which can then be utilized by the electron transport chain to produce ATP through OXPHOS. Inhibition of CPT1 α by etomoxir has been shown to significantly impact the survival of regulatory T cells (T_{reg}) [14], leading to speculation that FAO is required for T_{reg} maintenance and generation. However, etomoxir can have off target effects unrelated to fat oxidation [15], and most of the studies on T_{reg} and FAO analyzed T_{reg} generation following prolonged *in vitro* culture. Furthermore, inhibition of fat oxidation did not block human inducible T_{reg} generation [16], suggesting that the full impact of fat oxidation on T_{reg} development and function await further investigation.

Regulation of enzymes and metabolites in both the TCA and FAO pathways are critically important to understanding T cell metabolism, and the reader is encouraged to seek out multiple detailed reviews published recently on this subject [32–34]. To briefly summarize the TCA cycle and its enzymes, acetyl-CoA, generated by either FAO or glycolysis, is joined to oxaloacetate by citrate synthase to form citrate. Citrate is then converted to isocitrate by

aconitase, which is further processed to α -ketoglutarate by isocitrate-dehydrogenase (IDH). Processing of α -ketoglutarate by α -ketoglutarate dehydrogenase to form succinyl-CoA is followed by formation of succinate by succinate thiokinase. Succinate is reduced by succinate dehydrogenase to fumarate which is processed by fumarase to form L-malate. Finally, L-malate is reduced by malate dehydrogenase (MDH) to form oxaloacetate, completing the cycle.

To date, little work has analyzed the effect of specific TCA cycle enzyme inhibition on T cell proliferation and function. However, recently LW6, a putative HIF-1 α inhibitor, was shown to specifically target malate dehydrogenase-2 (MDH2), blocking the oxidation of malate and reducing NADH and FADH₂ generation [17]. LW6 was then used to interrogate the effect of MDH2 inhibition on T cell proliferation and apoptosis. Blockade of MDH2 *in vitro* reduced T cell proliferation, decreased apoptosis, and mediated metabolic adaptations to compensate for increased energy loss [18]. Another TCA cycle enzyme linked to T cells is isocitrate dehydrogenase 2 (IDH2). Mutations in IDH2 are found in angioimmunoblastic T cell lymphoma, where mutated IDH2 catalyzes transformation of isocitrate to 2-hydroxyglutarate, an oncogenic metabolite that alters histone methylation [19], in a process that is similar to what is observed in some forms of acute myelogenous leukemia. Future insights into the role of TCA cycle enzymes and T cell function may result from detailed observation of individuals with germline mutations in genes such as fumarase [20] and succinate dehydrogenase [21], or from the study of T cell function following exposure to specific enzyme inhibitors.

As previously described, activated T cells undergo rapid metabolic reprogramming that engages both glycolytic and oxidative metabolism. Adoption of these specific metabolic pathways is controlled by a rapid increase in the expression of transcription factors which drive bioenergetic programs. One such transcription factor is the proto-oncogene Myc, which is known to upregulate a host of genes involved in glycolysis [24]. Activation of Myc, in response to TCR signaling, has been understood for many years to be responsible for T cell proliferation [22, 23], and Myc was recently shown to be responsible for promoting both glycolysis and glutaminolysis in activated T cells [25]. HIF-1 α , another transcription factor critical is heavily involved with T cell development, proliferation, and differentiation [27], also drives T cell glycolysis [26].

To upregulate oxidative metabolism, immune cells utilize several transcription factors including peroxisome proliferator activated receptor (PPAR) family and its co-factors (e.g. PPAR γ co-activator 1- α) and PPAR family members exert significant effects on T cell development and function [29], activities likely married to their metabolic regulation. For example, PPAR- α drives transcription of genes in FAO pathways including CPT1 α , fatty acid binding protein 4 (FABP4) and FABP5 [28]. Sterol regulatory element binding proteins (SREBP) are additional transcription factors that control lipid metabolism, often by influencing cholesterol handling and regulating control of FAO [30]. SREBPs reprogram lipid metabolism during T cell activation, and aid in the clonal expansion of cells in response to antigenic stimuli, but do not participate in homeostatic proliferation [31]. That said, there are few studies directly linking changes in lipid metabolism to anti-cancer responses, and when lipid metabolism is involved in T cell responses, e.g. following allogeneic HSCT, we

will highlight these findings in greater detail. For the remainder of this review, when oxidative metabolism is referred to, it will conceptually include any lipid metabolism that generates ATP through the TCA cycle and/or subsequent OXPHOS.

While the classic paradigm of activated T cells utilizing glycolysis provides a conceptual framework for understanding T cell metabolism, significant caveats exist. Many of the classic experiments on immunometabolism were performed on cells cultured and activated *in vitro*, where bioenergetic substrate such as glucose, glutamine, and oxygen are supplied in extra-physiologic amounts [27, 35]. In addition, metabolic pathways do not always represent an “either/or” scenario and T cells can upregulate both aerobic glycolysis and oxidative metabolism simultaneously, following either *in vitro* and *in vivo* activation. There is speculation that these processes serve distinct purposes, for example with OXPHOS necessary for early phase proliferation, while glycolysis supports T cell effector functions, specifically interferon gamma production [36]. Similar findings exist in alloreactive T cells during graft-versus-host disease, where activated cells rapidly acidify their media *ex vivo*, but also robustly increase their oxygen consumption rates (personal observation). Finally, proteins like AMP-activated protein kinase (AMPK), a cellular energy sensor known to drive fat oxidation in multiple systems, is upregulated in effector T cells [37], where the classic paradigm suggests glycolysis would predominate. These findings all suggest that metabolic adoption of glycolysis versus oxidative metabolism is not an “all or none” event and that metabolic reprogramming is likely more nuanced and context dependent than is commonly assumed.

It should be mentioned that metabolic substrates beyond glucose, oxygen and fatty acids are required during T cell proliferation and function. Amino acids, specifically, have been studied for their role in promoting essential T cell functions and this data is reviewed nicely elsewhere [38]. Briefly, glutamine, arginine, and leucine are all nutrients required during activation and early effector function. T cells cultured *ex vivo* are highly sensitive to the glutamine concentration of the media and T cell receptor (TCR) activation leads to an upregulation of glutamine transporters and increase in glutamine import [39]. This increased uptake is regulated, at least in part, by the transport protein Slc1a5, as absence of Slc1a5 decreases glutamine transport and impairs T helper (Th) 1 and Th17 responses [40]. T cell activation also causes large decreases in intracellular L-arginine levels, and supplementation of the media with excess arginine increases T cell survival and enhanced cytotoxicity against tumor cells *in vivo* [41]. Finally, the role of leucine in T cells was elucidated through study of the neutral amino acid transporter Slc7a5. During activation, T cells upregulate expression of Slc7a5 and absence of this transporter renders cells unable to clonally expand, metabolically reprogram, or differentiate in response to antigen, findings that were later shown to be a result of poor leucine uptake [42].

The remainder of this review will step away from the broader aspects of T cell metabolism and instead focus on the metabolic pathways present in T cells during their interactions with various cancers and tumor microenvironments. Both positive and negative effects of modulating T cell metabolism in these contexts will be considered and speculation given on ways that targeting metabolic pathways might improve cancer treatments and overall clinical outcomes.

Allogeneic T cells activate mitochondrial metabolism

Hematopoietic stem cell transplantation (HSCT) from an allogeneic donor is a curative treatment for leukemia, lymphoma, and related hematologic malignancies. During this procedure, the patient's native immune system is ablated by chemotherapy and/or irradiation, followed by infusion of donor bone marrow or peripheral blood cells to facilitate immune reconstitution [43]. The major side-effect of allogeneic HSCT is graft-versus-host disease (GVHD), where alloreactive T cells from the graft recognize host antigens as foreign and mount a pathologic immune response against host tissues. GVHD can be eliminated by depleting T cells prior to infusion, but this modification results in higher rates of fatal infection and increased rates of relapse given that graft-versus-tumor (GVT) responses are eliminated [44]. GVHD prophylaxis with calcineurin inhibitors block T cell proliferation and cytotoxic potential [43], but these therapies are not universally protective and 30–50% of patients still develop acute GVHD. Steroids remain a first-line of therapy for acute GVHD, but only 50% of patients respond [45] and steroid refractoriness portends a poor prognosis [46]. Those that do respond, but remain on steroids, are susceptible to infection, hypertension, hyperglycemia and a range of steroid-related side effects [47]. Post-transplant patient care thus becomes a delicate balance between limiting GVHD, maximizing GVT effects, and preventing the development of new pathologies. Recent advances in understanding T cell metabolism may hold the key to separating GVHD from GVT responses and unlocking the full potential of HSCT as an anti-cancer therapy.

Alloreactive T cells, in contrast to relying exclusively on glycolytic metabolism, also upregulate OXPHOS, as exemplified by both increased oxygen consumption and decreased levels of intracellular pyruvate [48]. Alloreactive T cells also significantly increase fatty acid uptake and upregulate CPT1 α expression, an enzyme critical for mitochondrial lipid oxidation. Consistent with the importance of mitochondrial metabolism to alloreactive T cells, inhibition of CPT1 α with etomoxir reduces alloreactive T cell proliferation and mitigates GVHD severity [49]. Using a similar model, Glick et al. showed that alloreactive T cells activated *in vivo* predominantly increase oxidative metabolism, with glycolytic metabolism remaining more prominent during allo-activation *in vitro*, again highlighting the difference between *in vitro* and *in vivo* environments and underscoring the importance of defining the contextual landscape of metabolic studies [50]. Despite a wealth of data indicating the importance of OXPHOS and FAO in alloreactive T cells, some controversy remains. A recent study analyzing the metabolic profile of alloreactive cells determined glycolysis to be predominant [51]. While it is difficult to fully reconcile these separate lines of evidence, several explanations may account for the disparity. The latter study relies heavily on transcriptional and metabolic profiling which, while useful, provide only a snapshot of events and do not indicate flux of metabolites over time. Earlier studies are careful to use functional readouts of metabolism including parameters such as ^{13}C -palmitate conversion to metabolic intermediates *in vivo* or the functional oxidation of fat *ex vivo*. Differences in mouse strain combinations may also account for some discrepancies, but data on human T cells during xenogeneic GVHD supports an increase in both fat oxidation and glycolysis, consistent with the earlier findings [52].

In addition to understanding the eventual outcome of T cell metabolic reprogramming, it is equally important to define the molecular and transcriptional changes that control this process. While significant progress has been made in correlating the metabolic phenotype of T cells with different phases of activation, knowledge of the molecular mechanisms underpinning these changes lags behind. Recent research has revealed that signaling through the negative regulator Programmed Death-1 (PD-1) can influence T cell metabolism. Ligation of PD-1 on T cells increases beta-oxidation of fatty acids and actively represses glycolysis [53], with a similar phenomenon found in alloreactive T cells. Here PD-1 signaling increases the rate of mitochondrial oxygen consumption and T cell survival. Conversely, PD-1 blockade in alloreactive cells decreases fat oxidation and lowers intracellular levels of reactive oxygen species (ROS), which at least partially explains the decreased apoptosis observed following PD-1 or PDL-1 antagonism [52].

Given that alloreactive T cells adopt a metabolism distinct from cells undergoing homeostatic reconstitution [49], this may open the door for selective suppression of GVHD-causing T cells with preservation of homeostatic and additional immune responses including GVT effects. In this way, metabolic targeting of transcription factors, energy sensors, and enzymes might significantly impact antileukemia therapies, not by modulating the leukemia response itself, but rather by reducing the toxicity of an otherwise effective therapy. Considering this, there are many transcription factors that regulate OXPHOS, including the PPAR family, which are activated during starvation or times of high energy need. PPAR α is downregulated during GVHD, but other family members may be responsible for regulating FAO, and blocking PPAR γ has been shown to reduce alloreactivity [54]. In a similar vein, PPAR γ coactivator 1 alpha (PGC1 α), a transcriptional coactivator and major regulator of mitochondrial biogenesis, is upregulated in alloreactive T cells, suggesting a mechanism for the enhanced oxidation of fat [49]. AMPK, a well-studied energy sensor becomes, activated during times of starvation and upregulates OXPHOS to increase energy production [55]. However, in CD4 T cells, AMPK has been suggested to skew differentiation towards a regulatory phenotype through control of fat oxidation [56], a finding at odds with a proposed role for AMPK in promoting T_{eff} responses [37]. Thus, it remains unclear whether blockade or enhancement of AMPK signaling might be the best therapeutic approach. Sirtuins are a family of deacetylase enzymes and SIRT1 is known to regulate OXPHOS by activating AMPK, PPARs and PGC1 α [57] and has also been shown to impact metabolism in a variety of T cell-mediated inflammatory contexts [58, 59]. These findings provide a therapeutic rationale for studying the role of SIRT1 in GVHD pathology. Many of the gene and protein targets listed above have yet to be tested *in vivo* and must further be assessed for their ability to discriminate between GVHD and preservation of homeostatic and anti-cancer functions. The continuing hope is that the robust activation experienced by alloreactive T cells leads to metabolic changes that leave them susceptible to selective metabolic inhibition, thereby improving the therapeutic window of HSCT, which remains a mainstay of anti-cancer therapy.

Metabolic reprogramming to enhance anti-cancer therapies: Leveraging metabolism to enhance T cell responses: CAR T cells

In the previous section, the discussion focused on modulating T cell metabolism to limit pathogenic immune responses. In other contexts, it may be advantageous to maximize T cell responses, as in the case of chimeric antigen receptor (CAR) T cells responding against target antigens on cancer cells [9]. In their simplest form, CARs consist of an extracellular antigen-recognition domain (commonly an antibody single-chain variable fragment), a transmembrane domain, and an intracellular sequence for signal transduction, most commonly the CD3 ζ chain. Upon antigen recognition and ligation of the extracellular domain, T cells become activated resulting in proliferation, cytokine secretion and cytolytic activity. First generation CARs, consisting of only antigen recognition and CD3 ζ domains, faced significant challenges including a lack of cytotoxicity against target cells and minimal persistence in the host [60, 61]. Inclusion of costimulatory domains in 2nd and 3rd generation CAR constructs have improved their overall efficacy [62] [63], resulting in complete response rates of >70% using CAR T cells with reactivity against CD19 expressed on B cell malignancies [64]. However, despite promising results for CD19-reactive CARs, targeting of other antigens, particularly in solid tumors, has been less successful, and active efforts are underway to improve these approaches [62, 64]. Here, we consider what is known about the role of metabolism in CAR T cell therapy, and discuss ways to improve CAR T cell efficacy through metabolic manipulation.

In multiple CAR treatment scenarios, loss of CAR T cells from the circulation portends a negative prognosis with often imminent relapse of the underlying malignancy [65–67]. Furthermore, it is well established that persistence of transferred lymphocytes correlates with cancer regression [68] and this sets up the argument that increasing T cell persistence should increase the efficacy of anti-cancer therapies, with modulation of CAR T cell metabolism as a potential means of prolonging CAR T cell persistence.

Most of the recent clinical successes using CAR T cells have come from incorporation of the CD28 and 4–1BB costimulatory domains into 1st generation CAR constructs [69]. Although both costimulatory domains appear to elicit similar response rates in patients with refractory ALL [69, 70], data is emerging that these constructs likely achieve results in different ways. Inclusion of the CD28 domain leads to higher levels of early T cell proliferation, with a more rapid elimination of cancer in the first 7 days, followed by waning of the T cell response. In contrast, incorporation of the 4–1BB domain leads to noticeably slower T cell accumulation and tumor regression kinetics [71], but increased CAR T cell persistence in both mice and humans [67, 69, 71]. In some cases, decreased persistence of CD28-bearing CARs has been attributed to tonic signaling of the CAR construct itself, leading to an exhausted phenotype with increased expression of TIM-3 and LAG-3 [72]. However, the CD19-reactive CAR T cells do not suffer this same fate for reasons which remain unclear.

Interestingly, the costimulatory domain of the CAR construct also appears to track with the metabolic profile of the CAR T cell (Figure 1). Second-generation CARs bearing a 4–1BB costimulatory domain show higher rates of basal oxygen consumption, an increase in spare respiratory capacity, and enhanced conversion of ¹³C-palmitate into Acetyl-CoA, a hallmark

of beta-oxidation. This increased oxidative potential correlates with an increase in mitochondrial biogenesis, as evidenced by both more mitochondria per cell and a larger percent of cell area occupied by mitochondria. This has been corroborated *in vitro*, where activated CD8 T cells stimulated with a 4-1BB agonist antibody increase fatty acid uptake [73] and where 4-1BB containing CAR T cells, after 9 days in culture, enrich for multiple genes involved in cellular metabolism [72]. In contrast, CD28-containing CAR T cells produce lactate at a faster rate, an indirect measure of glycolytic potential, and demonstrate increased mRNA expression of multiple glycolytic genes including glucose transporter 1 (Glut1) and pyruvate dehydrogenase kinase 1 (PDK1) [74]. It is perhaps not surprising that expression of a CD28 signaling domain tracks with increased glycolysis, as CD28 signaling in naive T cells activates PI3K and AKT and has been shown to facilitate glucose uptake and increase glycolytic flux [3]. As an aside, lactate overproduction can have negative effects on T cell cytolytic activity, which may partially explain the suboptimal results seen with CD28-containing CARs [75]. To compound this challenge, lactate effects tumor cells by promoting of angiogenesis, migration and metastasis, enhancing radiosurvival, and increasing immune evasion [76]. Thus, it is theoretically possible that CD28-containing CARs, through an increase in glycolysis and elevated production of lactate, might promote a pro-tumor response. However, because CD28-containing CAR T cells show limited persistence *in vivo* (diminished as early as 4 weeks post-transfer) [63], they are unlikely to serve as a long-term, lactate-producing reservoir to promote tumor growth.

From the above studies, a general pattern emerges where glycolysis favors early proliferation but decreased long-term persistence, while OXPHOS correlates with increased persistence and memory formation. If true, the metabolic question going forward becomes how to best balance proliferation versus persistence to optimize CAR T cell efficacy [64]. If rates of glycolysis are too high, T cells are likely to exhaust, persistence will fall, and responses are unlikely to be maintained. If OXPHOS predominates, early responses may be suboptimal, with the potential for tumor escape and less effective therapy overall. Several approaches may be envisioned to overcome these challenges. Limiting glucose concentrations during *ex vivo* expansion may keep cells from becoming overdependent on glycolysis [7, 50], while activating T cells in the presence of a glycolytic inhibitor (e.g. 2-deoxyglucose), or impairing Glut1 expression during CAR T cell generation, may accomplish a similar function [77]. Additional *ex vivo* manipulation, such as treating cells with glycogen synthase kinase 3- β inhibitors, might not only promote memory stem cell formation but may also de-repress AMPK, further facilitating an oxidative phenotype [78]. Finally, as suggested by others, optimal anti-tumor responses may be obtained by simultaneous transfer of both CD28- and 4-1BB-bearing CAR T cells, where CD28 CARs will provide an immediate but short-lived response, while 4-1BB CARs will provide delayed but persistent, anti-tumor activity [79] (figure 1). Ongoing clinical trials using 3rd generation receptors (NCT01853631), in which a CD19-targeting CAR will contain both CD28 and 4-1BB moieties, may also help address this question.

Finally, the necessity of expanding CAR T cells *ex vivo* provides a unique opportunity to introduce additional changes to shape their future metabolic potential prior to re-infusion. For example, it is theoretically possible to transduce key metabolic regulators directly into CAR T cells (e.g. AMPK), and thus control the metabolic phenotype of the responding cells.

Conversely, clustered regularly interspaced short palindromic repeat (CRISPR) technology [80, 81], or lentiviral driven expression of short hairpin RNA, might be used to decrease expression of proteins in pathways we wish to demote (e.g. Glut1), and thus steer cells away from metabolic profiles shown to be less advantageous *in vivo*. Breakthroughs in this area, however, will only come through a detailed and nuanced understanding of the pathways and metabolic regulators necessary for activation and persistence of T cells *in vivo*, a process which is only just beginning.

T cell metabolism in solid tumors

In the previous section, we discussed the role of metabolism and its ability to influence whether T cells effectively target leukemia and other blood cancers. In solid tumors, the contextual landscape affecting T cell metabolism and function are arguably more complex. Extrinsic factors in the tumor microenvironment, limitations on nutrient availability, and crosstalk amongst a variety of cells, including the tumor cells themselves, all impact T cell functions and have been reviewed in depth elsewhere [82]. In this section, we focus specifically on T cell metabolism and how it might be manipulated to improve anticancer treatments against solid tumors.

As detailed above, activated T cells engage in aerobic glycolysis to facilitate proliferation and execute effector functions. Thus, when solid tumors consume large amounts of glucose, this may create a glucose-deficient environment [83], limiting the amount of glucose available to local anti-tumor T cells, causing metabolic insufficiency and limiting their effector function. This raises the intriguing possibility that anti-tumor immunity might paradoxically be improved through therapeutic manipulation of tumor cell metabolism. Indeed, this has been shown in a mouse model of sarcoma, where blocking PD-L1 directly on tumors inhibits mTOR activity, decreasing glycolytic enzymes and dampening tumor-associated glycolysis. This metabolic change returns glucose to the microenvironment, where it can be used by local T cells, resulting in the return of effector cytokine production [84]. Conversely, increasing glucose utilization in normally “regressing” tumors, and thereby stripping the T cells of their glucose supply, is enough to override the local immune response and leads to unrestrained tumor growth. Given that immune checkpoint blockade facilitates durable clinical responses against a growing list of cancers [85], it will be interesting in future studies to pair availability of intratumoral nutrients with improvements in clinical outcomes.

In addition to changing metabolism within tumor cells, it may be also possible to change metabolism directly within the T cell, and thereby facilitate changes despite the low extracellular glucose levels. Increased expression of the enzyme phosphoenolpyruvate carboxykinase (PCK1), in an adoptive transfer model, increases conversion of oxaloacetate to phosphoenolpyruvate (PEP), a glycolytic intermediate [86]. However, in addition to simply raising rates of glycolysis, PEP also facilitates Ca^{2+} -driven NFAT signaling by repressing Ca^{2+} uptake by the endoplasmic reticulum-associated sarco/ER Ca^{2+} -ATPase, in the process increasing effector function.

In the absence of sufficient glucose, it is hypothetically possible for tumor-infiltrating T cells to rely on OXPHOS to maintain their metabolic needs. However, recent work in a B16 mouse melanoma model shows that tumor infiltrating lymphocytes (TILs) also decrease oxidative metabolism, as indicated by smaller mitochondrial mass, depressed oxygen consumption, and limited spare respiratory capacity [87]. In this model, it was postulated that chronic AKT signaling in anti-tumor cells drives progressive loss of PGC1 α expression, with a subsequent decrease in mitochondrial biogenesis and lowering of oxidative potential. Constitutive expression of PGC-1 α by retroviral transduction increased oxygen consumption rates and facilitated effector cytokine expression in intratumoral T cells. Furthermore, injection of PGC1 α over-expressing, tumor-specific T cells into tumor-bearing mice in an aggressive tumor model prolonged survival and led to a higher incidence of complete regression (Figure 2, Top).

Recent work in a model of clear cell renal cell carcinoma (ccRCC) complements these findings, demonstrating impairment of both glycolysis and dysregulation of mitochondrial metabolism in CD8⁺ TILs [88]. CD8 TILs from ccRCC primary tumors decreased their proliferation to anti-CD3 stimulation and expressed lower levels of the activation markers CD25 and CD71. These changes correlated with decreased glucose uptake and suggested a decreased utilization of glycolysis. In contrast to the B16 model, however, T cells from ccRCC patients demonstrated an increase in mitochondrial mass, with hyperpolarization of the mitochondrial membrane. Confocal microscopy, however, demonstrated fragmented mitochondrial morphology and a large increase in mitochondrial reactive oxygen species (ROS), suggesting significant dysregulation of mitochondrial function. Importantly, addition of pyruvate to CD8⁺ TILs during *ex vivo* anti-CD3 stimulation restored CD25 and CD71 expression, suggesting that correction of metabolic impairments may improve tumor responses, data which adds to a growing list of examples where manipulation of metabolic pathways directly impacts the direction and intensity of T cell responses [36, 89].

The above evidence indicates that the metabolic program of a T cell is intimately linked to its anti-tumor activity and consequently, tumor growth and patient survival. Manipulating tumor-specific T cell metabolism may thus be a promising approach to improve current therapies, but targeting metabolism directly in tumor-resident T cells is exceedingly difficult. One way to potentially overcome this challenge is through the use of adoptive cell therapy (ACT) [90]. This approach utilizes both naturally occurring, and bioengineered, T cells, which are expanded *ex vivo* and may be represented by TILs, CAR T cells, or cells of a donor lymphocyte infusion (DLI). Isolated T cells would then be metabolically manipulated, for example through transduction of a metabolic driver, to optimize their cytolytic activity prior to reinjection into the patient [7]. (Figure 2, Bottom).

While it is certainly feasible to manipulate cells by constitutive expression of metabolic proteins, a simpler approach may be to culture cells in conditions that favor oxidative metabolism and/or select for cells post-expansion whose metabolic profile produces the most metabolically fit T cells *in vivo*. Indeed, one recent study showed that segregating T cells based upon low mitochondrial membrane potential selected for cells with increased FAO, greater self-renewal, and enhanced persistence and anti-tumor activity *in vivo* [91]. In a similar light, culturing cells in media supplemented with additional L-arginine shifted cell

metabolism from glycolysis to oxidative phosphorylation and endowed transferred T cells with higher survival capacity *in vivo* and enhanced anti-tumor activity [41]. This work shows that it is possible to select metabolically fit cells *ex vivo* for enhanced performance *in vivo*. This idea of “preprogramming” cells metabolically appears to be a general phenomenon across cell types, as isolating and processing hematopoietic stem cells under physiologic oxygen concentrations (which are much more hypoxic than ambient 21% O₂), yields many more cells and with better engraftment compared to cells processed in room air [92]. Putting these findings together, it is easy to conceptualize future protocols where cells destined for ACT are handled under strict metabolism-promoting culture conditions, selected for future metabolic fitness *in vivo* based on defined *ex vivo* cell parameters, and introduced into the patient. As stated elsewhere, success in these endeavors is fundamentally dependent upon determining the most effective culture conditions and pre-injection parameters for achieving the desired immune-response *in vivo*, a process that is ongoing.

Therapeutic targeting of metabolic pathways

Until this point, our attention has focused on the metabolic changes within T cells, how these changes are impacted by environmental factors (e.g. immunosuppressive tumor bed), and whether decreases (to curb GVHD) or increases (to improve *in vivo* T cell persistence) in metabolism might positively impact anticancer outcomes [7, 54]. In this section, we briefly highlight metabolic changes in the tumor cells themselves and then explore potential therapeutic approaches with compounds that might have ‘dualaction’, wherein they can positively impact T cell responses while still reducing tumor growth.

Allogeneic HSCT may be the setting where dual-action therapeutics prove to be most efficacious. Ideally, pre-transplant conditioning clears the large majority of leukemic cells, with transplanted T cells eradicating any remaining tumor cells through a GVT effect. Unfortunately, too often leukemic cells escape clearance, resulting in early disease recurrence, a situation particularly true for patients who enter transplantation with clear signs of minimal residual disease [93–95]. In this scenario, adjuvant therapies which mitigate GVHD while simultaneously decreasing leukemia burden would be particularly helpful. It has been established that oxidative metabolism represents a viable therapeutic target in alloreactive T cells to reduce the severity of GVHD. In this light, it is interesting to consider recent metabolic findings in leukemia stem cells (LSC). In patients with acute myelogenous leukemia (AML), primary cells with the highest rates of engraftment (and thus presumed to be LSCs), had low ROS levels, an increased dependence on OXPHOS, and increased BCL-2 expression at both the protein and mRNA level. [96]. This is particularly interesting as BCL-2 has recently been shown to play a noncanonical role in regulating oxidative and mitochondrial metabolism in tumor cells [97]. Furthermore, exposure of primary AML cells to the BCL-2 inhibitor AB-263 impaired their oxidative potential, while treating tumor xenografts with the related small molecule inhibitor ABT-737 decreased engraftment of primary human AML cells [96]. ABT-737 has also been used to treat GVHD in animal models, where ten days of administration decreased GVHD clinical scores and significantly enhanced survival [98]. A direct effect of BCL-2 inhibition on alloreactive T-cell metabolism was not examined, so it is possible that ABT-737 is working through distinct mechanisms to inhibit alloreactive T cells versus leukemia stem cells. However, it is

striking that a compound known to target oxidative metabolism is effective against two cell types with an increased dependence on OXPHOS [48, 96]. From a clinical standpoint, BCL-2 inhibition demonstrated little clinical efficacy in early trials [99], but ABT-263 has shown some success in recent trials [100] and further work is anticipated to determine the effects of BCL-2 inhibition on the metabolism of alloreactive T cells. Finally, BCL-2 inhibition also enhances *in vitro* killing of malignant B-cells by CAR T cells [101]. While some of this enhanced cytotoxicity appears to be due to proapoptotic effects directly on leukemia cells, in lines resistant to apoptosis, ABT-737 treatment still provides some enhancement of killing, suggesting that ABT-737 may work directly on the CAR T cells themselves. These findings require *in vivo* corroboration and confirmation of a mechanistic link from BCL-2 inhibition to metabolism in alloreactive cells, but certainly warrant further study.

Blockade of FAO represents a second potential target for dual-action therapies [102, 103]. During beta-oxidation, CPT1 α couples Acyl-CoA moieties to carnitine, allowing their transport into the mitochondria for subsequent oxidation. As previously mentioned, CPT1 α protein and mRNA levels both increase in alloreactive T cells during GVHD, and inhibition of CPT1 α by etomoxir reduces alloreactive T cell proliferation and decreases disease severity [49]. Interestingly, inhibition of CPT1 α by the small molecule ST1326 also decreases fatty acid oxidation and increases apoptosis in both leukemia cell lines and primary leukemia samples [104] and perhexiline, a CPT1 α inhibitor approved for use in Australia [105], has shown anti-leukemia effects [106, 107]. The difficulty with many of these compounds is their serious side effect profiles [108], which for etomoxir includes cardiac hypertrophy and for perhexiline involves both neuro- and hepatotoxicity [109, 110]. Potential solutions to this challenge are to identify common drivers upstream of FAO in alloreactive T cells and tumors that might be targetable, or alternatively to look for compounds that block FAO in novel ways. Avocatin B, a lipid derived from the avocado fruit, is one example of a novel compound. Avocatin B blocks fatty acid oxidation, and reduces NADPH levels, in primary human leukemia cells, resulting in ROS-dependent cell death [111]. This cytotoxicity depends upon both mitochondrial localization of Avocatin B and functional expression of CPT1 α . Although the impact of Avocatin B on alloreactive T cells remains untested, the selectivity of Avocatin B towards AML versus normal hematopoietic stem cells suggests that this compound warrants a closer look as a potential dual-action inhibitor following allogeneic HSCT.

Summary/Conclusion:

T cells experience large swings in energy demand during the transition from naïve cells, to highly proliferative effectors, and back to quiescent memory cells. During this course, metabolic processes are tightly regulated to meet energetic outputs, and perturbations in this balance can affect not only rates of T cell proliferation, but also influence differentiation and effector function. Given the degree of these dramatic changes, and the fact that different metabolic pathways are active in different contexts, there exists a large window of opportunity for therapeutic targeting of metabolism in pathogenic T cells, in the case of GVHD, or alternatively enhancing cells that are underperforming, in the case of CAR T cells and TILs. This review has highlighted recent research that defines the energy pathways

adopted by T cells involved in anti-cancer responses and suggested ways that these adaptations might be leveraged to enhance anti-cancer therapies. In an ideal scenario, metabolic interventions will be selected that have specific effects on both immune cells and the cancer cells to which they are responding, thus improving therapeutic efficacy and overall patient outcomes. Future work requires the elucidation of which metabolic pathways are most critical to efficient T cell responses *in vivo*, should explore how these pathways are regulated, and will evaluate novel approaches to target these pathways, to improve future anti-cancer therapies.

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- Discuss our current understanding of T cell metabolism during activation and GVHD
- Review recent progress in understanding the metabolism of TIL and CAR T cells
- Discuss metabolic approaches to target cancer cells and enhance immune function

Figure 1. Evolution of chimeric antigen receptors adds domains that regulate T cell metabolism and enhance *in vivo* performance.

First generation CARs contained only a single CD3 ζ signaling domain and exhibited poor performance *in vivo*. Addition of the CD28 domain activates glycolysis, allowing greater T cell proliferation and target cell killing. Inclusion of 4-1BB domains enhances OXPHOS in T cells increasing proliferation as well as persistence and memory. 3rd generation CAR constructs containing both domains are currently under evaluation in clinical trials, with an expectation of further improved performance.

Figure 2. Metabolic paradigms for enhancing *in vivo* performance of T cells.

Top: Tumor specific T cells transduced with a PGC1 α transgene enhance mitochondrial biogenesis. Introduction of modified T cells into tumor-bearing hosts increased proliferation, persistence and target cell killing [87]. **Bottom:** Applying a similar concept, tumor-specific T cells, harvested from tumor samples, engineered with CARs, or isolated from a donor lymphocyte infusion (DLI) sample, might be metabolically reprogrammed by enhancing or inhibiting a variety of protein targets. These changes will promote greater metabolic sufficiency of the cells and increase T cell persistence after introduction to the host, potentially improving patient outcomes.