



The role of fatty acid oxidation in the metabolic reprogramming of activated T-cells

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Activation represents a significant bioenergetic challenge for T-cells, which must undergo metabolic reprogramming to keep pace with increased energetic demands. This review focuses on the role of fatty acid metabolism, both *in vitro* and *in vivo*, following T-cell activation. Based upon previous studies in the literature, as well as accumulating evidence in allogeneic cells, I propose a multi-step model of *in vivo* metabolic reprogramming. In this model, a primary determinant of metabolic phenotype is the ubiquity and duration of antigen exposure. The implications of this model, as well as the future challenges and opportunities in studying T-cell metabolism, will be discussed.

Keywords: T-cell metabolism, fatty acid oxidation, oxidative phosphorylation, reactive oxygen species (ROS), AMP-activated protein kinase, graft-versus-host disease, *in vivo* models

INTRODUCTION

Activation precipitates a dramatic change in T-cell physiology. Upon stimulation, T-cells increase their DNA replication, synthesize cytokines, and upregulate multiple signaling pathways (1, 2). Proliferation increases exponentially, with stimulated cells dividing as frequently as every 4–6 h at the height of an immune response (3, 4). The energetic requirements for these new tasks dictate that T-cells must undergo metabolic reprogramming in order to generate sufficient biomass and produce adequate adenosine triphosphate to meet the increased metabolic demands (5).

In recent years, increasing attention has focused on the metabolic pathways adopted by T-cells following activation. Many fine contemporary reviews highlight the relationship between metabolic phenotype and signal transduction (6, 7), T-cell differentiation (8, 9), and T-cell function (5, 10). Other reviews stand as thorough summaries on overall T-cell metabolism, and the reader is encouraged to seek out these important works (11). This review will focus on the use of fatty acid oxidation (FAO) by activated T-cells, both *in vitro* and *in vivo*, and suggest a possible connection between the environment present during activation and adoption of this alternative metabolic pathway. To place the findings on FAO into a contextual framework, I will begin by briefly reviewing the role of other metabolites, including glucose and amino acids, in T-cell metabolism.

THE NECESSITY OF GLYCOLYSIS AND AMINO ACIDS

Early studies demonstrated increased rates of both glycolysis and lactate production during mitogen activation of rat thymocytes, suggesting a prominent role for glucose metabolism during *in vitro* T-cell stimulation (12). Following activation, T-cells increase multiple steps in glucose metabolism, including upregulation of the glucose transporter Glut1, in a highly regulated process that is at least partially dependent upon signaling through the co-stimulatory molecule CD28 (13–15). Failure of

T-cells to sufficiently increase glucose metabolism decreases both proliferation and cytokine production, while overexpression of a transgenic Glut1 receptor increases cytokine production and improves T-cell survival (16, 17).

Glutamine metabolism is also requisite during T-cell activation and limiting glutamine in the culture media decreases proliferation and cytokine production in mitogen-stimulated lymphocytes (18). Studies on purified populations of T-cells confirmed the importance of glutamine uptake during *in vitro* stimulation and implicated a role for CD28 in maximizing glutamine uptake (19). In addition, inflammatory CD4 T-cell responses depend on glutamine uptake through expression of the amino acid transporter Slc1a5 (20), and absence of Slc1a5 decreases the percentage of IFN- γ ⁺ T-cells responding to *Listeria monocytogenes* infection. Similarly, the transcription factor Myc plays a pivotal role in directing glutamine into obligate biosynthetic pathways and facilitates the initial proliferative burst (21). Thus, both glucose and glutamine appear indispensable for early events in T-cell metabolic reprogramming.

In addition to glutamine, T-cells require access to other amino acids for proliferation and survival. Expression of the bidirectional glutamine/leucine transporter Slc7a5 is an integral event in early T-cell activation and absence of this receptor decreases T-cell responses both *in vivo* and *in vitro* (22). The importance of this receptor is intriguing, given that leucine is a necessary component of T-cell activation and that higher glutamine levels facilitate leucine import through simultaneous glutamine export (23). Therefore, a large role for glutamine may simply be to provide an intracellular gradient to support transport of other amino acids. This hypothesis is supported by the finding that glutamine transporter deficiency can be overcome through increasing concentrations of leucine. Additional data suggest that glutamine transport may even initiate metabolic adaptation, as absence of Slc1a5 in T-cells blunts expression of other metabolic mediators including both Glut1 and CD71 (22).

In addition to leucine, T-cells also depend upon tryptophan to execute full effector function. Suppressed T-cell responses are observed when antigen presenting cells contain indolamine 2,3-dioxygenase (IDO), an enzyme that catabolizes tryptophan (24, 25). The importance of the IDO pathway has been demonstrated in multiple immunogenic processes, including fetal tolerance during pregnancy, bone marrow transplantation, antitumor responses, and autoimmunity (26). In addition, kynurenine, a tryptophan catabolite, induces regulatory T-cell generation (T_{reg}) through its action on the aryl-hydrocarbon receptor (21). Thus, T-cell responses can be modulated by both decreased levels of a nutrient (tryptophan) and the actions of its metabolic derivative (kynurenine).

NUTRIENT REGULATION OF T-CELL DIFFERENTIATION

Other nutrients also influence T-cell differentiation and function. Short-chain fatty acids, such as propionate and butyrate, are generated via fermentation by intestinal bacteria and intestinal levels of these fatty acids also modulate T_{reg} formation (19, 27, 28). Similar to kynurenine, propionate and butyrate likely drive T_{reg} formation through specific intestinal T-cell nutrient receptors, but the precise mechanism has yet to be confirmed. In a similar way, $V\alpha 9^+V\delta 2^+$ gamma-delta T-cells selectively respond to the microbial metabolite (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (29). High salt concentrations also affect T-cell function and drive CD4 T-cells toward a Th17 phenotype both *in vivo* and *in vitro*. This is clinically relevant because dietary increases in salt worsen the severity of experimental autoimmune encephalomyelitis in murine models (30, 31). Salt sensitivity in Th17 cells occurs via increased expression of serum glucocorticoid receptor-1, a protein, which governs sodium homeostasis in multiple cell types (32). Thus, extracellular nutrients not only help meet increased energy needs of activated T-cells, but may also dictate their differentiation and effector status.

THE ROLE OF FATTY ACID OXIDATION

Oxidation of fat, in addition to the catabolism of glucose and glutamine, was first implicated as an energy source in unstimulated lymphocytes (33), although most studies suggest that naïve T-cells require only a minimal rate of metabolism to meet their bioenergetics needs (5, 34). A role for FAO in other subsets first came from work in T-cells bearing a deletion of TNF receptor associated factor 6 (TRAF6). CD8 T-cells deficient in TRAF6 are unable to form memory cells in response to infection with *L. monocytogenes* and when taken *ex vivo*, decrease rates of β -oxidation (35). Furthermore, indirect activation of AMP-activated protein kinase (AMPK), a cellular energy sensor and controller of FAO (36–38), increased CD8 memory T-cell generation and improved survival in a lethal tumor model. Subsequent *in vitro* work demonstrated that IL-15, an important cytokine in memory T-cell generation, upregulates expression of carnitinepalmitoyltransferase 1a (CPT1a), the rate limiting enzyme in FAO (39). These studies suggest a subset specific role for FAO in the generation of CD8 memory T-cells.

CD4 T-cells cultured *in vitro* also exhibit a subset specific dependence on FAO. T-cells differentiated *in vitro* toward Th1, Th2, or Th17 profiles adopt a glycolytic phenotype, consistent with earlier findings on T-cell metabolism (13, 14). In contrast,

T_{regs} generated *in vitro* increase lipid oxidation and phosphorylate AMPK. Furthermore, *in vitro* blockade of FAO with the CPT1a inhibitor etomoxir disrupts T_{reg} generation and *in vitro* supplementation with fatty acids supports T_{reg} function (17). *In vivo* administration of metformin increases both the percentage and total number of T_{reg} during a murine model of asthma (17).

Together, these data support a mandatory role for FAO in both IL-15 driven CD8 T-cell responses and in the induction of *in vitro* generated T_{regs} . Furthermore, metformin administration, which indirectly activates AMPK, increases both T_{reg} and memory CD8 T-cells, and could indicate a role for AMPK in controlling FAO in these cell types (17, 35). This notion is supported by the fact that CD8 T-cells deficient in AMPK α 1 mount inferior memory T-cell responses following *L. monocytogenes* infection (40). It remains unclear, however, exactly how metformin increases T_{reg} and CD8 memory T-cells and the extent to which AMPK controls pathways of T-cell metabolism beyond FAO (41–43).

Metformin is a direct inhibitor of Complex I of the electron transport chain (44) and through inhibition of oxidative phosphorylation can indirectly accelerate glycolysis (45). However, it is unlikely that increased glycolysis drives memory T-cell formation, as glycolytic inhibition has already been shown to increase CD8 memory T-cell generation (46). However, the rapid recall response of memory T-cells requires an imprinted glycolytic potential (47), suggesting that the transition from memory to effector phenotype depends upon glycolysis and is, therefore, potentially influenced by the indirect effects downstream of metformin. Further clarity on the direct role of AMPK in driving FAO and will be gained using more selective inhibitors of AMPK and genetically deficient animal models (43, 48).

One of the key challenges in studying immune cell metabolism *in vitro* is the relevance of experimental systems to the environmental conditions encountered *in vivo* (10, 49). Standard culture concentrations differ greatly from physiologic values, including higher concentrations of glucose (three- to fivefold higher than the standard serum glucose of 5 mM), glutamine (eightfold higher than serum levels), and oxygen (21% in culture compared to 2–5% *in vivo*) (50). Changes in these environmental variables can affect both a cell's function and metabolic response (51). For example, decreased glucose availability modulates both oxygen consumption and metabolic transcription factors during human CD4 T-cell activation (52). Hypoxia reduces proliferation and cytokine production and promotes glycolysis (53, 54). Thus, the metabolic phenotype adopted by a T-cell following *in vitro* stimulation may be very different from the phenotype adopted by T-cells activated under physiologic conditions *in vivo* (55).

One attractive approach to this challenge is to study metabolic reprogramming in lymphocytes activated *in vivo* during graft-versus-host disease (GVHD). During GVHD, alloreactive donor T-cells respond robustly to the presence of host antigens, leading to marked proliferation, destruction of host tissues, and profound inflammation (56–58). Allogeneic T-cells taken directly from GVHD animals demonstrate a 2.5-fold increase in oxygen consumption (a surrogate for oxidative phosphorylation) and a modest increase in the expression of Glut1 (59). Increased oxidative metabolism during GVHD is consistent with data from patients with systemic lupus erythematosus, where isolated T-cells increase

their mitochondrial mass by 50% and expand their mitochondrial membrane potential by 20%. Similarly, peripheral blood mononuclear cells from these patients increase oxygen consumption by 50% compared to healthy control cells (60, 61).

Recent studies suggest a direct role for FAO in effector T-cells during GVHD. Levels of acylcarnitines, necessary intermediates in the oxidation of fat (62), increase 10-fold or more in allogeneic T-cells by day 7 post-transplant (59). In addition, effector GVHD T-cells (characterized by their CD44^{hi}, CD62^{Lo} phenotype) increase fatty acid transport, upregulate levels of CPT1a and CPT2, and increase their rates of FAO *ex vivo* (63). Treatment of allogeneic cells with etomoxir selectively decreases their proliferation *in vitro*, while etomoxir administration *in vivo* decreases both total donor T-cell numbers and the severity of GVHD (63). In contrast, T-cells proliferating in a homeostatic fashion after transplantation, and those responding to cellular immunization, minimally increase fatty acid transport and display no sensitivity to etomoxir (63). Thus, FAO appears to be a specific metabolic adaptation in effector T-cells proliferating in response to large quantities of antigen. This is supported by findings in an experimental autoimmune encephalomyelitis model, where etomoxir blockade of FAO decreased disease severity, limited demyelination, and reduced effector cytokine production (64).

Consistent with an increase in oxidative phosphorylation, allogeneic T-cells also increase generation of reactive oxygen species (ROS) (59). Increased ROS likely result from increased mitochondrial membrane potential ($\Delta\psi_m$), which prolongs the half-life of reactive intermediates in the electron transport chain, leading to increased leak of single electrons from the intra-mitochondrial space and subsequent formation of ROS (65). The increased ROS observed in allogeneic T-cells is also consistent with data from patients with systemic lupus erythematosus, where T-cells exhibit both hyperpolarization of the $\Delta\psi_m$ and increased ROS (61). In addition, increases in ROS and oxidative phosphorylation can be therapeutically targeted, as modulators of complex V of the electron transport chain mitigate the severity of GVHD without affecting homeostatic reconstitution (59).

The increased glycolysis in T-cells during GVHD is modest compared to the level of glycolysis and glucose uptake observed during *in vitro* activation (14, 55). Calculations based upon O₂ consumption suggest a larger contribution from oxidative metabolism toward total energy production in allogeneic T-cells (66). This disparity might be accounted for by differences between *in vitro* and *in vivo* conditions, as described earlier (52). However, this explanation fails to account for situations where *in vivo* effector T-cells do not upregulate fatty acid transport following cellular immunization or increase mitochondrial mass following infection, as might be expected from cells with increased oxidative metabolism (39, 63). To unify the disparate results both between *in vitro* and *in vivo* conditions, and across different *in vivo* scenarios, I propose a multi-step model of metabolic reprogramming in T-cells (Figure 1), where a primary determinant of metabolic phenotype is both the duration and degree of environmental stimulation present at the time of analysis.

In setting up this model, it is important to note that T-cells during GVHD do not increase fatty acid transport until their fifth division, which occurs on approximately day 3 post-transplant

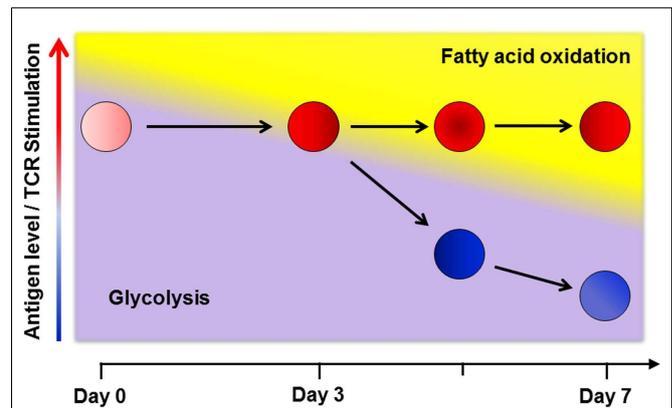


FIGURE 1 | A multi-step model of *in vivo* metabolic reprogramming. Early in a graft-versus-host (GVH) response, T-cells use glycolysis, glutaminolysis and glucose oxidation to meet their short term energy needs (16, 21, 67). By day 3 post-transplant, robustly activated cells (shown in red) require additional metabolic reprogramming to keep pace with ongoing energetic demands and so upregulate fatty acid oxidation (FAO) by increasing fat uptake, turning on co-activator molecules, and upregulating fatty acid oxidation enzymes. This transition comes with a concomitant rise in reactive oxygen species and a moderation in the rate of glycolysis (63, 68). In contrast, T-cells stimulated via cellular immunization, with a limited duration of antigen exposure, only transiently increase fat uptake and ROS on day 3. As antigen levels fall, stimulation decreases and cells no longer require FAO (cells shown in blue). Levels of oxidation enzymes, co-activator molecules, fat transport, and ROS levels decrease to baseline in these cells (63). Thus, despite similar CD44^{hi}CD62^{Lo} effector profiles, the metabolic phenotype on day 7 is clearly different between robustly and transiently activated T-cells. From these data, I propose that a primary determinant of metabolic reprogramming in effector T-cells is both the degree and duration (< or >3 days) of antigen exposure at the time of evaluation.

(63). Thus, early events in T-cell activation, even in the presence of significant antigen, do not require additional fatty acids. Instead, early during a graft-versus-host (GVH) response, T-cells likely utilize glycolysis and glutaminolysis to meet their short-term energy needs (16, 21). This idea is supported by data obtained following *in vivo* administration of the superantigen staphylococcal enterotoxin B (SEB), where SEB sensitive CD4⁺, V β 8⁺ cells undergo a 15-fold increase in glycolysis 48 h post-administration of SEB (21). Initial dependence on glycolysis also explains the early *in vivo* sensitivity of T-cells to the glycolysis inhibitor 2-deoxyglucose (67).

Later in the response (i.e., after 4–5 cell divisions), robustly activated T-cells require additional reprogramming to keep pace with the ongoing demands of persistent activation (68). This reprogramming includes increased fatty acid uptake, upregulation of oxidation enzymes and co-activator molecules, moderation in the rate of glycolysis, and adoption of FAO with a concomitant rise in ROS. This view is consistent with effector T-cells maintaining oxidative phosphorylation following activation under a variety of activating conditions (21, 39, 69). Mechanisms that drive this second metabolic transition remain undefined, but signaling through PD-1 is known to restrict glycolysis (70). In addition, AMPK promotes FAO in multiple systems and is known for its ability to act as a “cellular energy sensor” (36, 37, 71, 72). Indeed, knock-out of AMPK α 1 increases Glut1 expression, hexokinase levels,

and glycolytic metabolism in purified T-cells. These observations suggest that when present, AMPK might actively dampen T-cell glycolysis (73), perhaps at the cost of promoting FAO (Figure 2).

Implicit in the multi-step model is the idea that continued stimulation of the T-cell receptor (TCR) drives later stages of metabolic reprogramming. As antigen levels fall during the resolution of an immune response, stimulation decreases, energetic demands shrink, and effector T-cells no longer require utilization of alternative energy sources. Transgenic OT-I T-cells, when transferred into irradiated recipients that bear ovalbumin as a self-protein, markedly increase fat transport, ROS levels, and markers of oxidative metabolism. In contrast, when the same OT-I T-cells are stimulated by immunization with OVA-bearing dendritic cells, they return to baseline values of fat transport and oxidative metabolism by day 6 post-immunization (63). In these models, differences between GVHD and immunization responses were not dependent on differentiation status of the responding OT-I cells. Cells from either environment had similar CD44^{Hi}CD62^{Lo} profiles and made equivalent amounts of IFN-gamma upon re-stimulation, consistent with the ability to separate effector function and metabolic phenotype on a per cell basis (74).

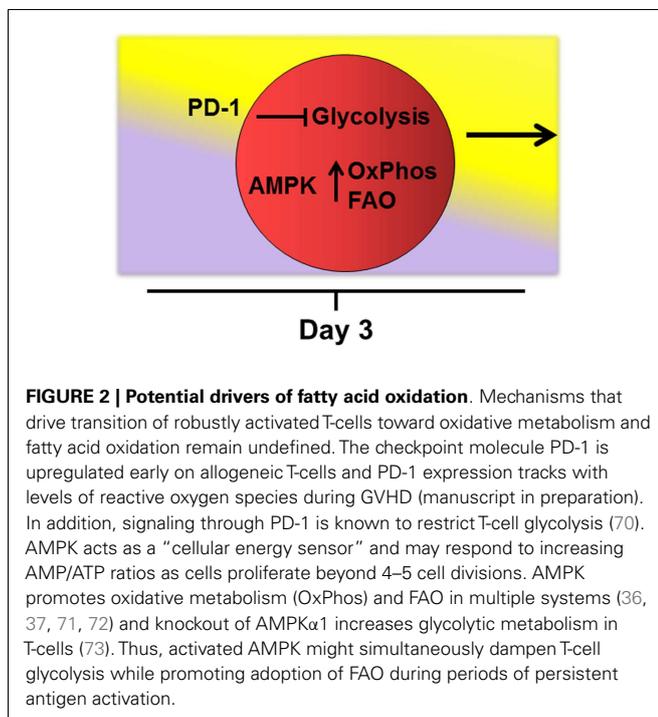
Although allogeneic T-cells only modestly increase glycolysis (59), glucose is still likely being utilized by these cells in alternative pathways. Indeed, shunting of glucose through the pentose phosphate pathway increases levels of reducing equivalents through production of NADPH and also generates building blocks for nucleic acid synthesis (75). In addition, glucose derivatives can be combined with oxaloacetate in the TCA cycle to form citrate, exported back to the cytosol via the carnitine/palmitate shuttle, converted into acetyl-CoA and then malonyl-CoA via the action of acetyl-CoA carboxylase 1 (ACC1), and eventually incorporated into *de novo* lipid synthesis. Evidence that a similar process

occurs in activated T-cells comes from work using ACC1 deficient T-cells. CD8 T-cells lacking ACC1 have impaired survival following *L. monocytogenes* infection (74) and ACC1 deficiency preferentially disrupts *in vitro* differentiation of Th17 T-cells while simultaneously sparing development of T_{reg} (76). This Th17/T_{reg} dichotomy is reminiscent of studies on hypoxia-inducible factor 1- α and aryl-hydrocarbon receptor signaling, where promotion of Th17 responses occurs at the expense of T_{reg} generation (77, 78). In addition, both treatment with an ACC1 specific inhibitor and use of ACC1 deficient T-cells leads to diminished severity of EAE (76). However, in contrast to FAO inhibition, which preferentially affects antigen-activated T-cells (63), deficiency of ACC1 impacts both antigen-activated and homeostatic responses (74). This suggests that lipid synthesis, as driven by ACC1, is likely a necessity for proliferation of all T-cells.

Similar to the diversity of glucose metabolism, glutamine may, in addition to glutaminolysis, play a role as a metabolic substrate in one-carbon serine metabolism, as has been shown for proliferating cancer cells (79). Thus, as effector T-cells proliferate beyond 4–5 cell divisions with ongoing TCR stimulation, the roles of glucose and glutamine likely change, as increased fat transport feeds fatty acids into the TCA cycle. However, even fat-derived intermediates may exit the TCA cycle into alternative metabolic shunts (e.g., via the aspartate-malate shuttle) and the role of these divergent pathways during the latter stages of ongoing T-cell activation remain exciting areas for future investigation.

Given the ability of effector T-cells to reprogram their metabolic phenotype, the question arises as to whether T-cells can be pre-programmed for the physiologic conditions they will encounter *in vivo*, a potential advantage when providing anti-viral or anti-tumor immunity. Recent work lends credence to this possibility. Treatment of *ex vivo* CD8 T-cells with the glycolysis inhibitor 2-deoxyglucose (2DG) increases phosphorylation of AMPK, heightens oxygen consumption, and decreases multiple markers of glycolysis in these cells. In addition, *ex vivo* treatment of cells with 2DG enhances their *in vivo* antitumor function (46). These data suggest that glycolytic inhibition *ex vivo* drives upregulation of alternative metabolic phenotypes, which then provide a subsequent selective survival advantage *in vivo*.

In contrast to allogeneic activation, T-cells responding via homeostatic proliferation minimally upregulate fatty acid transport and are not susceptible to FAO inhibition. This observation suggests that the metabolic demands of homeostatic renewal are distinct from those of T-cell activation, as has been seen in other studies of T-cell metabolism (22). These results also imply that modulating T-cell metabolism may offer a selective intervention against pathogenic cells, potentially leading to a decrease in overall immunosuppression (55, 59). From this perspective, it becomes critically important to understand at which stage of metabolic reprogramming the intervention occurs. Some therapies, such as antithymocyte globulin, will eliminate all T-cells regardless of proliferation or activation status. Other treatments, like inhibition of ACC1, will affect survival of all proliferating T-cells, regardless of the stimulus (antigen-activated versus homeostatic cues) (74). Interventions that disrupt early events in TCR-activated metabolic reprogramming (e.g., disruption of amino acid transport) might spare homeostatic T-cells, but will target T-cells whose metabolic



reprogramming is driven through TCR stimulation (80). Finally, interventions that affect the latter stages of metabolic adaptation, such as inhibition of FAO or modulation of oxidative phosphorylation, will likely only inhibit effector T-cells responding to prolonged antigenic stimulus (63). This last form of intervention may be particularly relevant in T-cells undergoing continuous exposure to antigen (e.g., during autoimmunity and following transplantation of bone marrow or solid organs) and highlights situations that will gain the most from selective immunotherapy, as current immunosuppression for these disorders leads to significant morbidity and mortality (81–84).

CHALLENGES FOR THE FUTURE

The rapid increase in our understanding of T-cell metabolism offers exciting opportunities and presents several challenges. The majority of initial metabolic studies were performed *in vitro* and many of these paradigms and results may not reflect *in vivo* biologic reality, which needs to be addressed. In addition, metabolic adaptation is by necessity a dynamic and responsive process, and conditions both inside and outside the T-cell change dramatically from one moment to the next. Thus, a thorough view of metabolism in any model needs to incorporate data and observations from multiple time points of analysis. Third, we know very little about the molecular machinery that drives adoption of metabolic phenotypes, particularly *in vivo*. Follow-up studies will need to not only identify proteins important in T-cell metabolism, but also define how the dependence on these factors changes during the course of an immune response. The field also needs metabolic activators and inhibitors with increased specificity, both for study purposes and the potential for therapeutic intervention (43). Finally, the study of T-cell metabolism needs to expand to better include the human immune system, particularly in the context of immune-mediated disorders. Future studies in human beings will benefit both from clinically based flux analysis using labeled metabolites such as ^{13}C -glucose or ^{13}C -palmitate (85, 86) and the great variety of pathways and compounds being discovered in the field of cancer therapy (79, 87–90).

CONCLUSION

T-cell activation represents a time of significant energetic stress and cells must respond to this challenge by reprogramming their metabolism to keep pace with increased metabolic demands. During a murine model of GVHD, effector T-cells increase their dependence on oxidative metabolism and FAO. Adoption of these pathways is likely due to environmental factors present at the time of T-cell recovery and analysis, including the ubiquity and duration of antigen exposure. Thus, T-cell differentiation status (e.g., memory versus effector) is not the sole arbitrator of metabolic phenotype, and our data suggest that effector T-cells will instead respond as necessary to meet the metabolic demands placed upon them, including upregulation of FAO. Future studies will determine how broadly findings on allogeneic T-cells can be applied to other models of chronic antigen exposure. Finally, these hypotheses must be tested in human immune responses, where a better understanding of T-cell metabolism might lead to enhanced vaccine strategies, improved anti-cancer responses, novel interventions against autoimmunity, and better post-transplant immunotherapy.

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