Daniel R. Wahl
Craig A. Byersdorfer
James L. M. Ferrara
Anthony W. Opipari Jr
Gary D. Glick

Distinct metabolic programs in activated T cells: opportunities for selective immunomodulation

Authors' addresses

Daniel R. Wahl[1], Craig A. Byersdorfer[2], James L. M. Ferrara[2], Anthony W. Opipari Jr[3], Gary D. Glick[1,4]

[1]Chemical Biology Doctoral Program, University of Michigan, Ann Arbor, MI, USA.
[2]Department of Pediatrics, University of Michigan, Ann Arbor, MI, USA.
[3]Department of Obstetrics and Gynecology, University of Michigan, Ann Arbor, MI, USA.
[4]Department of Chemistry, University of Michigan, Ann Arbor, MI, USA.

Correspondence to:

Gary D. Glick
Department of Chemistry, University of Michigan
930 N. University Avenue
Ann Arbor, MI 48109-1055, USA
Tel.: +1 734 764 4548
Fax: +1 734 615 8902
e-mail: gglick@umich.edu

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Summary: For several decades, it has been known that T-cell activation in *vitro* leads to increased glycolytic metabolism that fuels proliferation and effector function. Recently, this simple model has been complicated by the observation that different T-cell subsets differentially regulate fundamental metabolic pathways under the control of distinct molecular regulators. Although the majority of these data have been generated in vitro, several recent studies have documented the metabolism of T cells activated in vivo. Here, we review the recent data surrounding the differential regulation of metabolism by distinct T-cell subsets in vitro and in vivo and discuss how differential metabolic regulation might facilitate T-cell function vis-à-vis proliferation, survival, and energy production. We further discuss the important therapeutic implications of differential metabolism across T-cell subsets and review recent successes in exploiting lymphocyte metabolism to treat immune-mediated diseases.

Keywords: *lymphocyte, metabolism, oxidative phosphorylation, glycolysis, reactive oxygen species, Bz-423*

Classical metabolic model of T-cell activation

In the absence of stimulation, the primary function of T cells is antigenic surveillance, which requires relatively small amounts of energy in the form of adenosine triphosphate (ATP), to support cellular processes such as ion homeostasis (1, 2). Initial studies in unstimulated rat thymocytes and lymphocytes suggested that glucose, fatty acid, and glutamine oxidation supply ATP in these cells (3–5). The simultaneous measurement of oxygen consumption and lactate production revealed that unstimulated thymocytes generate 96% of their ATP via oxidative phosphorylation (OXPHOS) with glycolysis only providing 4% (6). More recent studies have observed a similar metabolic phenotype in unstimulated mouse and human T cells (7, 8), suggesting that the dependence on OXPHOS is a generalizable feature of resting lymphocyte populations (9).

Following activation, cellular responses ensue, including transcriptional program activation, cytokine synthesis and

secretion, and rapid proliferation. These functions require increased ATP and metabolic precursors for biomass synthesis (1, 10). How activated lymphocytes meet these increased metabolic demands has been an active area of investigation for nearly 50 years (11). Rat thymocytes activated in vitro for 2–3 days increase rates of glycolysis and lactate production by 20- to 50-fold compared with unstimulated cells (6, 12, 13). Similar increases in glycolysis are observed when mouse and human T cells are activated in vitro (7, 8). Interestingly, activated T cells and thymocytes do not increase tricarboxylic acid (TCA) cycle oxidative activity to nearly the same extent as glycolysis (6, 13, 14). Rather, the majority of glucose metabolized is released as lactate (12), with most of the remainder entering the pentose phosphate cycle (PPC) (3, 14) or otherwise being incorporated into biomass (15) (Fig. 1). Removing glucose inhibits T-cell proliferation and cytokine production, even when other metabolic substrates such as glutamine or fatty acids are present, likely due to the ability of glucose metabolism to concordantly generate ATP and NADPH and stabilize anti-apoptotic proteins (8, 16). Although less is known about the metabolism of activated B cells, they appear to utilize high rates of glycolysis much like activated T cells (17).

The increased glycolysis that occurs with the activation of mouse and human T cells in vitro is accompanied by only a twofold increase in OXPHOS, as measured by mitochondrial oxygen consumption (7, 14). This increased OXPHOS is almost certainly not fueled by fatty acid oxidation (FAO), as in vitro stimulated mouse T cells decrease palmitate oxidation up to sevenfold compared with unstimulated T cells (14, 18). Rather, the modest increase in OXPHOS in activated T cells in vitro is likely fueled by the oxidation of glutamine in the TCA cycle, which increases fourfold in mitogen-stimulated rat thymocytes or antibody-stimulated mouse T cells (12, 14). The importance of glutamine metabolism for activated T-cell function is emphasized by the observation that elimination of glutamine from culture media decreases the proliferation and cytokine production of mitogen-stimulated rat and mouse lymphocytes (19, 20). Interestingly, the OXPHOS inhibitor myxothiazol does not inhibit proliferation or cytokine production in human CD4$^+$ T cells stimulated with PMA and ionomycin (21). This observation suggests that the importance of increased glutaminolysis for activated T-cell function is due to its ability to facilitate biomass synthesis rather than its ability to fuel oxidative ATP production (22). However, when glucose is removed from the media, myxothiazol becomes a potent inhibitor of T-cell proliferation and cytokine production, suggesting that mitochondrial ATP production plays an important role in activated T-cell function when rates of glycolysis are low (21).

Upregulation of glycolysis in the presence of oxygen has been termed the Warburg effect or aerobic glycolysis due to its initial characterization in tumor cells by Otto Warburg in the early 20th century (10, 23). Aerobic glycolysis is a relatively inefficient pathway to generate ATP from glucose, as complete oxidation of glucose to CO_2 produces at least
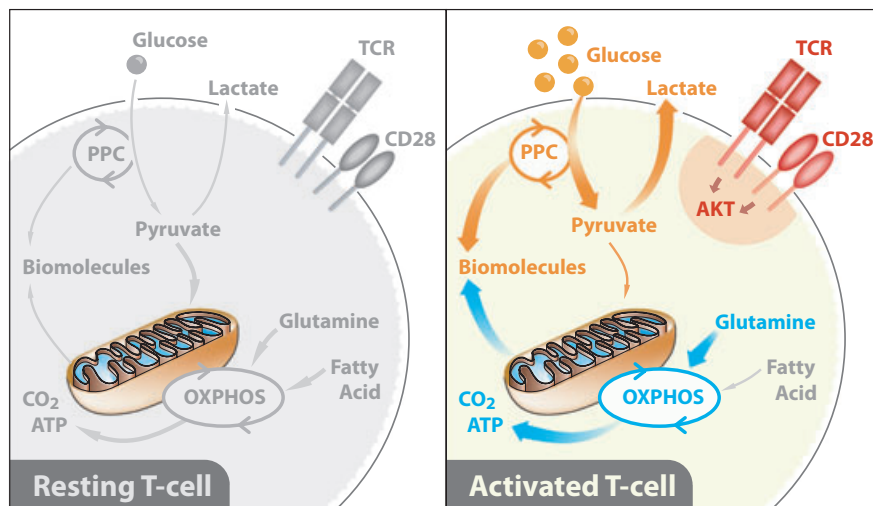

Fig. 1. A simple metabolic model of T-cell activation. Resting T cells (left) take up glucose and secrete lactate at low rates. Their lack of proliferation coincides with a low rate of biomolecule synthesis. Their metabolic demands are met by a low rate of oxidative phosphorylation (OXPHOS) fueled by the oxidation of glucose, glutamine, and fatty acids. Activated T cells (right) dramatically upregulate glycolysis and lactate production. Biomass synthesis is facilitated by the action of the pentose phosphate cycle (PPC) as well as the partial mitochondrial metabolism of glucose and glutamine. Although OXPHOS is increased, fatty acid oxidation decreases compared with resting T cells. These changes were initially thought to be primarily due to the CD28-dependent activation of AKT.

15-fold more ATP than metabolism to lactate (10). However, many types of proliferating cells rely on aerobic glycolysis as, in addition to ATP synthesis, it produces reducing equivalents for pathways that detoxify oxygen radicals and provides precursors for biomass synthesis (10, 13, 24).

T-cell activation requires engagement of the T-cell receptor (TCR) and costimulation through CD28. CD28 signaling activates the kinase AKT (7, 8) (Fig. 1), which was thought to drive increased glycolysis (1) due to its ability to increase the expression and surface localization of the glucose transporter GLUT1 (7, 8) and increase the activities of key glycolytic enzymes (25–28). Indeed, inhibiting AKT signaling with inhibitors of phosphatidylinositol-3-kinase (PI3K) prevents activated T cells from upregulating glycolysis (7). In other cell types (e.g. adipocytes, fibroblasts, and immortalized lymphoid cells), AKT signaling stimulates OXPHOS and fatty acid synthesis, two pathways that are increased in activated T cells (29–32). Hence, increased AKT signaling could account for many of the metabolic changes observed in activated T cells *in vitro*. Signaling through inhibitory receptors on T cells [programmed death-1 (PD-1) or cytotoxic T-lymphocyte antigen-4 (CTLA-4)] decreases AKT activity and glycolysis (33). Hence, the inverse regulation of AKT by costimulatory and inhibitory receptors could explain how T cells match metabolic activity with function (1).

Expanding the model of T-cell metabolism to account for specific T-cell subsets

Activated $CD4^+$ T cells and effector $CD8^+$ T cells (so called because they have been appropriately stimulated to lyse target cells) both increase glycolysis following activation (7, 16, 34). However, effector $CD8^+$ T cells may not increase OXPHOS above resting cells (34), which distinguishes them from activated $CD4^+$ T cells that, as described above, increase OXPHOS by about twofold (7, 35). Consistent with their lack of increased OXPHOS, effector $CD8^+$ T cells are exquisitely sensitive to the availability of glucose: lowering glucose concentrations from 5 (physiologic) to 0.5 mM decreases effector $CD8^+$ T-cell proliferation by fourfold (36) and similar reductions decrease IFN-γ production by 80% (37). By contrast, $CD4^+$ T cells maintain their ability to proliferate and secrete cytokines at low levels of glucose (7, 8). These results point to metabolic differences between activated T-cell subsets with this particular example suggesting that $CD4^+$ T cells have greater metabolic flexibility with respect to energy-rich substrates than effector $CD8^+$ T cells.

Does the difference in substrate dependence manifest as differences between $CD4^+$ and $CD8^+$ T-cell function? Following pathogen clearance, antigen, costimulatory ligands, and activating cytokines decrease causing most activated T cells to decrease glycolysis and undergo apoptosis, while a small number transition to memory cells (38, 39). In this process, the activated $CD4^+$ T-cell population size contracts significantly slower than $CD8^+$ T cells, suggesting that activated $CD4^+$ T cells have a survival advantage over effector $CD8^+$ T cells when pro-glycolytic signaling is limited (40, 41). This observation could be due to the ability of $CD4^+$ T cells to upregulate OXPHOS when glycolysis is limited, allowing them to survive longer than effector $CD8^+$ cells that fail to increase OXPHOS.

Interestingly, the forced expression of active AKT promotes growth and survival in $CD4^+$ T cells but not $CD8^+$ T cells (42–44), suggesting that $CD4^+$ T cells may be more dependent on AKT for their metabolic phenotype than $CD8^+$ T cells. Indeed, inhibiting AKT in activated murine $CD8^+$ T cells with a pan-isoform inhibitor does not decrease glucose uptake but rather decreases the expression of numerous effector molecules (36). Hence, metabolic differences between activated $CD4^+$ and effector $CD8^+$ T cells could be due to distinct roles for AKT in these two cell types.

After activation, $CD4^+$ T cells differentiate into a number of subtypes: Th1 cells, which promote cell-mediated immunity; Th2 cells, which promote humoral immunity; Th17 cells, which promote mucosal immunity and inflammation; and regulatory T cells (Tregs), which inhibit immune responses. Interestingly, relative to unstimulated naive $CD4^+$ T cells, pro-inflammatory Th17 cells utilize higher rates of glycolysis, whereas anti-inflammatory Tregs increase FAO (45) (Fig. 2). Increased flux through these pathways provides an indication of their functional significance, which is supported by mechanistic experiments. For example, inhibiting glycolysis blocks Th17 cell differentiation *in vitro* and decreases disease severity in a Th17-mediated mouse model of experimental autoimmune encephalitis (EAE) (46). By contrast, the differentiation of Tregs is inhibited by the FAO inhibitor etomoxir but stimulated by glycolysis inhibition with 2-deoxyglucose (2DG) (45, 46).

Given these results, it is reasonable to postulate that the relative availability of glucose versus fatty acids as substrates within inflammatory microenvironments can control the nature of the immune response. Such metabolic microenvironments could provide an autoregulatory mechanism guarding against fulminant inflammatory responses, as the high consumption of glucose during acute immune

Cell Type	Metabolic Regulators	Principal Metabolic Pathways
CD4+ activated	Myc, ERR-α, pAKT	Glycolysis, OXPHOS
CD4+ Th17	HIF-1α	Glycolysis
CD4+ Treg	AMPK	Fatty Acid Oxidation
CD8+ effector	Myc, others?	Glycolysis
CD8+ memory	AMPK	Fatty Acid Oxidation

Fig. 2. Regulation of metabolic pathways in activated T-cell subsets. Activated CD4+ T cells initially dramatically upregulate glycolysis and modestly increase oxidative phosphorylation (OXPHOS). These changes are due to signaling through AKT, Myc, and estrogen-related receptor-α (ERR-α). Activated CD4+ T cells that differentiate into the Th17 lineage primarily rely on glycolysis that is controlled by hypoxia-inducible factor-1α (HIF-1α). By contrast, activated CD4+ T cells that differentiate into Tregs rely on fatty acid oxidation (FAO) under the control of AMP-activated protein kinase (AMPK). Effector CD8+ T cells principally upregulate glycolysis under the control of Myc, but other metabolic regulators remain unknown. Like Tregs, memory CD8+ T cells principally rely on FAO under the control of AMPK.

activation could deplete this substrate, which would induce Treg differentiation and the downregulation of inflammation.

The different metabolic patterns adopted by Th17 and Treg cells suggest differing mechanisms of metabolic regulation. T cells cultured in conditions favoring Th17 differentiation induce hypoxia-inducible factor-1α (HIF-1α) in a STAT3 (signal transducer and activator of transcription 3)-dependent fashion and the expression of HIF-1α is required for the induction of the Th17-defining molecules RAR-related orphan receptor γ (RORγ) and IL-17 (47). These results suggest that the pro-glycolytic effects of HIF-1α (48) might account for the increased glycolysis observed in Th17 cells. Indeed, the deletion of HIF-1α decreases glycolysis by fourfold in Th17 cells (46). Interestingly, HIF-1α shunts glucose-derived carbons away from the mitochondria by activating lactate dehydrogenase (LDH) and inhibiting pyruvate dehydrogenase (PDH) (49). This phenomenon limits biomass synthesis in favor of non-oxidative ATP production (15), and may contribute to the ability of Th17 cells to survive in hypoxic environments. Although its role in Th17 cells is clear, HIF-1α does not appear to be important for glycolysis in CD4+ or CD8+ T cells 1 day after activation, but may play a role at

later time points (14, 15), and little is known of its importance in Th1- or Th2-polarized cells.

Consistent with their dependence on FAO rather than glycolysis, the deletion of HIF-1α promotes rather than inhibits Treg differentiation, likely due to the ability of HIF-1α to bind to and degrade forkhead box P3 (47). Rather, Tregs express high levels of phosphorylated AMP-activated protein kinase (AMPK) (45), which regulates FAO by phosphorylating and inhibiting acetyl coA carboxylase (50, 51) thereby activating carnitine palmitoyl transferase 1a, the rate-limiting step in FAO (52). The importance of AMPK activation for the Treg lineage is supported by the observation that activating AMPK with metformin increases Treg differentiation in vitro, whereas its in vivo administration increases Treg numbers in a mouse model of asthma (45). A metabolic phenotype favoring FAO suggests that Tregs prioritize oxidative ATP production over the ability to generate biomass or generate ATP under hypoxic conditions, which could contribute to the stability of the Treg population in vivo (53).

Like Tregs and in contrast to effector CD8+ T cells, memory CD8+ T cells depend on AMPK-mediated FAO to meet their energetic demands (38, 39). Following clearance of antigen, growth factor signaling becomes limiting and a fraction of effector CD8+ T cells transition from a glycolytic metabolism that favors proliferation and biomass synthesis to oxidative ATP production that is required for long-term survival (38). This metabolic transition is stimulated by the pro-memory cytokine IL-15, which promotes mitochondrial biogenesis and the expression of CPT1 (39).

The importance of FAO for Tregs and memory CD8+ T cells has clinical implications. The administration of metformin, which activates AMPK and increases FAO, enhances memory CD8+ T-cell development after infection (38). Metformin also increases Treg responses while decreasing disease severity in a model of asthma (45). These findings highlight the potential to regulate metabolism as a therapeutic strategy to direct immune responses during infection, immunization, or inflammatory disease.

Several other regulators of activated T-cell metabolism have recently been discovered, although their differential importance among T-cell subsets remains unclear. In CD4+ T cells, estrogen-related receptor-α (ERR-α) is activated in a CD28-dependent fashion and is required for both increased glycolysis and OXPHOS (35). How ERR-α activates glycolysis and OXPHOS remains poorly understood; however, initial studies suggest that it may function to increase the activity of PDH (35). Myc becomes activated shortly after T-cell activation and is associated with the upregulation of

several key glycolytic enzymes including GLUT1, hexokinase, phosphofructokinase, and LDH (14, 54). Myc also induces glutaminolytic enzymes including glutaminase 2 and the glutamine antiporter CD98 (14). Deletion of Myc decreases glycolysis and glutaminolysis by nearly fourfold 1 day following T-cell stimulation in vitro (14). The importance of Myc-induced metabolic changes for activated T-cell function is emphasized by the observation that deletion of Myc abrogates the proliferation of both CD4$^+$ and CD8$^+$ T cells in vitro and in vivo (14). Additionally, the expression of Myc is necessary for the upregulation of PPC activity in activated T cells, implicating Myc signaling in the production of NADPH and biomass synthesis. Importantly, the deletion of Myc does not affect the expression of activation markers, suggesting that Myc abrogates activation-induced metabolic changes rather than blocking the entire activation signal (14).

Metabolic characteristics of T cells activated *in vivo*

Historically, nearly all of the work on lymphocyte metabolism employed primary immune cells cultured in vitro from several hours to several days, raising the possibility that in vitro culture affects the metabolic responses attributed to lymphocyte activation (3, 7, 55). For example, typical nutrient concentrations in plasma and culture media differ with respect to glucose (5 versus 10–25 mM) (7, 8, 56), glutamine (0.6 versus 2–4 mM) (57, 58), fatty acids such as oleate and palmitate (2–3 versus <20 μM) (58, 59), and oxygen (3–5% versus 20%) (60–62), and such differences in substrates can affect metabolic pathway utilization (7, 15). Indeed, OXPHOS activity in CD4$^+$ T cells and activated thymocytes changes when glucose levels are altered (6, 7, 21). However in non-lymphoid cells, supplementing culture media with oleate or palmitate to reach near-physiologic levels (0.25–0.5 mM) induces enzymes involved in FAO (63, 64) and decreases glycolysis (65). Finally, mouse T cells activated in vitro with levels of O$_2$ physiologic in conditions of active inflammation (1.5%) take up more glucose and secrete more lactate than cells grown under atmospheric O$_2$ (15). Together, these observations suggest that in vitro conditions have clear potential to influence lymphocyte metabolism. Thus, conclusions drawn based on studies involving protracted culture periods need to be interpreted within the context of the variables described above.

It is known that the nature and length of antigenic presentation and signal strength varies between in vitro and in vivo models (66). Depending on the nature of the antigen,

T-cell activation in vivo occurs with varied levels of chronicity (days to years), whereas stimulation in most in vitro models is on the order of hours to days (67, 68). Both CD4$^+$ and CD8$^+$ T cells downregulate glycolysis as they undergo repetitive stimulation in vitro (68), possibly due to decreased CD28 signaling and increased signaling through CTLA-4 and PD-1. Because the rate of glucose uptake in an activated T cell increases as the strength of antigenic stimulus increases (8), gauging qualitative or quantitative aspects of the metabolism of immune cells in vivo based on in vitro models depends on adequately understanding and controlling antigen affinity, T-cell avidity, and length of stimulation.

Recent developments in measuring oxygen consumption (69) and cell surface marker-based sorting (39) have facilitated studies of T cells activated in vivo. For example, the superantigen staphylococcal entertoxin B (SEB) activates Vβ8$^+$ T cells by crosslinking the Vβ8 subunit of the TCR directly to the major histocompatibility complex and activates the roughly 10% of T cells that are Vβ8$^+$ rather than the 1 in 10^5–10^6 that recognize an individual antigen (70–72). Two days following immunization with SEB, purified CD4$^+$ Vβ8$^+$ T cells underwent a 15-fold Myc-dependent upregulation of glycolysis compared with unstimulated CD4$^+$ Vβ8$^+$ T cells (14) (Fig. 3). Although this study did not address the effects on OXPHOS or any parameters of CD8$^+$ T-cell metabolism,

		OXPHOS	Glycolysis	Antioxidants
CD4$^+$ CD8$^+$	unstimulated	++	+	+++
CD8$^+$ Teff CD44^{hi} CD62L^{lo}	Listeria (6-7 days)	++	++	?
CD4$^+$ Vβ8$^+$	SEB (2 days)	?	+++++	?
CD4$^+$ CD8$^+$	GVHD (7 days)	++++	++	+

Fig. 3. Metabolic parameters of T cells activated in vivo. Effector CD8$^+$ T cells purified without regard to antigen specificity 6–7 days after Listeria monocytogenes infection have equivalent rates of oxidative phosphorylation (OXPHOS) as naive cells, but modestly increase glycolysis, and no information is available about their antioxidant levels. Activated Vβ8$^+$ CD4$^+$ T cells purified 2 days after staphylococcal entertoxin B (SEB) immunization increase glycolysis by 15-fold compared with unstimulated cells, but no information is available regarding their rate of OXPHOS or antioxidant levels. Proliferating donor T cells purified 7 days after the initiation of graft-versus-host disease (GVHD) increase OXPHOS 2.5-fold compared with unstimulated cells and modestly increase glycolysis. These cells suffer from the depletion of both glutathione and pyruvate.

the magnitude of the increase in glycolysis is similar to what is observed in in vitro models. Indeed, SEB stimulation shares several key features with in vitro models including a short time course, rapid clearance of the antigen, and robust activation of large numbers of T cells (73).

In other studies, a smaller increase in glycolysis was found activating CD8^{+} T cells in vivo. Endogenous CD8^{+} Teff cells (CD44^{hi}CD62L^{lo}) produced only 50% more lactate than naive CD8^{+} T cells (CD44^{lo}CD62L^{hi}) 6–7 days following infection with an attenuated strain of Listeria monocytogenes (39). Teff cells did not increase OXPHOS, consistent with in vitro studies (34). In this experiment, the population of Teff cells analyzed (CD44^{hi}CD62L^{lo}) was not antigen-specific and likely included T-cell clones of varying affinity for Listeria as well as bystander Teff with no relationship to Listeria. Hence, the heterogeneously activated Teffs may account for the relatively low rate of lactate production. In addition, as this analysis was done 6–7 days after bacterial infection, it is possible that the chronicity of exposure allowed downregulation of glycolysis (68).

Bioenergetic characteristics of disease-causing lymphocytes

Investigating the metabolism of lymphocytes mediating inflammatory diseases is challenging because these cells are very low in number, often cannot be identified directly, and are difficult to isolate from bystander cells in many disease models (74). With these limitations, many studies have characterized populations in which most cells are not pathogenic.

Notwithstanding these issues, potential metabolic differences related to disease have been identified. For example, peripheral blood T cells isolated from patients with lupus have 50% more mitochondrial mass than T cells from healthy volunteers, accompanied by a 20% increase in the mitochondrial membrane potential ($\Delta\psi_m$) (75). These findings are consistent with increased TCA cycle activity and OXPHOS and are supported by recent results showing that peripheral blood mononuclear cells (PBMCs) from patients with active lupus (and rheumatoid arthritis) consume 50% more O_2 than cells from healthy controls (76), and spleen tissue from mice with active lupus oxidize 40% more glucose in the TCA cycle than control cells (68). These data suggest that pathogenic lymphocytes upregulate OXPHOS, but the use of bulk cellular populations limits the conclusions that can be drawn.

In graft-versus-host disease (GVHD), alloreactive donor T cells proliferate in response to host antigen and mediate a potentially lethal immune response (77). In contrast to many autoimmune models, murine transplant models

employing congenic markers and cellular proliferation dyes allow disease-causing cells to be distinguished and isolated (67). Using these techniques, we observed that proliferating alloreactive donor T cells increase OXPHOS 2.5-fold compared with resting T cells 7 days after the initiation of GVHD (67) (Fig. 3). These GVHD-causing T cells also increase GLUT1 expression by 50% and lactate production fourfold in comparison with resting T cells (67), which is consistent with the increased uptake of a glucose analog observed in the intestinal tracts of mice and humans suffering from GVHD (78).

The increase in glycolysis observed in GVHD-causing T cells is modest compared with the increases seen in SEB-activated CD4^{+} T cells in vivo and CD8^{+} and CD4^{+} T cells activated in vitro. Notwithstanding the limitations imposed by assumptions regarding similar coupling efficiencies between cellular populations and the potential derivation of lactate from alternative pathways, the rates of oxygen consumption and lactate production can be used to estimate the proportion of ATP derived from OXPHOS and aerobic glycolysis, respectively (79). Such calculations suggest that, unlike T cells activated in vitro, alloreactive T cells rely primarily on OXPHOS for their synthesis of ATP (67).

Several questions arise in considering this unusual metabolic phenotype in GVHD-causing T cells. For example, why is their rate of glycolysis relatively low, and how are the proliferative demands for substrates necessary for biomass synthesis met in GVHD-causing T cells? During allogeneic transplantation, host professional antigen-presenting cells (APCs) stimulate donor T cells and propagate GVHD (80, 81). As some host APCs are not eliminated during GVHD (81), this ubiquitous presence of stimulatory alloantigen could result in the repetitive and chronic stimulation of GVHD-causing T cells. Recently, chronically stimulated T cells were observed to have two- to threefold lower rates of glycolysis than acutely activated controls (68), possibly due to decreased signaling through CD28 and increased signaling through PD-1 and CTLA-4 (68). The chronicity of stimulation during GVHD contrasts with activation by SEB, which falls to sub-stimulatory levels by 14 h after immunization and is completely cleared by 48 h (73). Hence, the acuity and rapid antigen clearance of the SEB model coupled with the time that measurements were made (2 days after SEB injection) may account for the high rates of glycolysis observed in activated T cells stimulated in vivo by SEB (14).

The molecular mechanism responsible for relatively low rates of glycolysis in GVHD-causing T cells remains unclear. Although increased signaling through PD-1 and CTLA-4

could play a role, we have found that despite their low rates of glycolysis, GVHD-causing T cells have high amounts of phosphorylated AKT at the time points in question (unpublished observations). Hence, signaling through PD-1 or CTLA-4 would have to be restricting glycolysis in an AKT-independent fashion. This scenario is most feasible for PD-1, which exerts its anti-glycolytic effects by inhibiting PI3K, which affects signaling molecules independent of AKT including the protein kinase C (PKC) family (33, 36). Regarding non-AKT regulators of metabolism, little is known about their activity in GHVD; microarray analysis has revealed that CD8^{+} effector T cells in murine GVHD have *decreased* levels of c-Myc mRNA compared to naive T cells (82). Hence, low rates of glycolysis in GVHD-causing T cells could be related to low activity of Myc. Given that members of the PKC family are known to regulate Myc levels (83), it is possible that chronic stimulation and activation of inhibitory receptors (e.g. PD-1) limits glycolysis in GVHD-causing T cells by inhibiting PKC and restricting Myc activity.

Proliferating cells must generate carbons precursors, reducing equivalents (NADPH) and free energy (ATP) to synthesize biomolecules, such as lipids, that are necessary to grow and divide. The T cells that cause GVHD rapidly proliferate with only a modest increase in glycolytic metabolism (67), so how do they supply metabolites necessary for biomass synthesis? GVHD-causing T cells are unlikely to be able to meet all three bioenergetic demands (NADPH, biomolecule precursors and ATP) using only their modest rates of glycolysis. However, unlike acutely activated T cells, there is evidence to suggest that GVHD-causing T cells upregulate FAO, a robust source of ATP (67). Indeed, GVHD-causing T cells have 10- to 20-fold increased levels of acyl-carnitines, an intermediate in FAO (67). Hence, GVHD-causing T cells might preferentially utilize glucose for NADPH and biomolecule precursor formation, while relying on FAO for ATP production. This hypothesis is especially appealing given the abundance of free fatty acids *in vivo* and their low levels in cell culture models. Further exploration is needed to determine the role of FAO during GVHD and if the regulators of FAO, such as AMPK, peroxisomal proliferator-activated receptor alpha (PPARα), and PPAR gamma coactivator-1 (PGC-1) contribute to the function of GVHD-causing T cells.

How would GVHD-causing T cells adopt this more efficient utilization of glucose? HIF-1α increases LDH expression and decreases the activity of PDH, thereby funneling glucose-derived carbons away from mitochondrial biomolecule synthesis (15). Indeed, the deletion of HIF-1α in activated T cells lowers lactate production and promotes glucose-derived carbon entry into the mitochondria for biomolecule synthesis (15), thereby facilitating proliferation. A recent microarray analysis showed that effector CD8^{+} T cells in a model of GVHD have threefold more HIF-1α mRNA than naive T cells (82), which is modest compared with the 15-fold increase in HIF-1α mRNA observed in T cells stimulated by anti-CD3 and anti-CD28 antibodies (84). Although the regulation of HIF-1α at the protein level cautions against the over-interpretation of mRNA levels (85, 86), these studies are consistent with an interpretation in which relatively low levels of HIF-1α activity allows GVHD-causing T cells to efficiently use glucose for biomolecule synthesis and proliferation rather than lactate production.

Redox consequences of altered bioenergetics in disease-causing lymphocytes

Although the mitochondrial electron transport chain (ETC) primarily functions to generate ATP, it is also the major source of reactive oxygen species (ROS) in mammalian cells, as 0.5–3% of the O_2 used by the mitochondria is converted into ROS in the form of O_2^- (87, 88). O_2^- generation occurs when single electrons 'leak' from reactive intermediates in the ETC (such as flavin mononucleotide or iron–sulfur clusters) and react directly with O_2 (89). Furthermore, as $\Delta\psi_m$ increases (termed 'hyperpolarization'), mitochondrial ROS production also increases, as hyperpolarization of $\Delta\psi_m$ prolongs the half-life of reactive intermediates and increases the likelihood that electrons react with O_2 to form O_2^- (90). Hence, cells increase ROS production as their utilization of OXPHOS increases.

Given that allo- and auto-reactive lymphocytes primarily rely on OXPHOS rather than glycolysis, it is not surprising that these cells exhibit increased ROS production. Indeed, compared with cells from healthy controls, T cells from patients with lupus exhibit both hyperpolarization of $\Delta\psi_m$ and increased ROS levels (91). Increased ROS production is also a characteristic of cells isolated from animals undergoing GVHD. Lymphocytes, neutrophils, and red blood cells from mice 5 weeks after GVHD induction have threefold higher levels of ROS compared with untreated control mice (92). Although these studies only examined bulk lymphocyte populations, our data indicate that GVHD-causing T cells also exhibit both hyperpolarization of $\Delta\psi_m$ and increased ROS levels (67).

The high rates of glycolysis associated with the Warburg effect benefit proliferating cells not only by allowing biomass and ATP production but also by facilitating antioxidant

production (10, 13). Glutathione is the most abundant anti-oxidant in mammalian cells and the formation of its active, reduced form requires NADPH (24). Inhibiting glycolysis with 2DG decreases total glutathione levels by 40% and induces dose- and time-dependent apoptosis in HeLa cells (93). This increased apoptosis is reversed by the antioxidant N-acetylcysteine, suggesting that it is due to increased ROS formation (93).

In addition to maintaining glutathione levels, glycolysis directly generates pyruvate, which acts as an antioxidant by eliminating H_2O_2 and reducing O_2^- production in mitochondria (94). The antioxidant functions of pyruvate are emphasized by experiments showing that the addition of pyruvate (0.1–2 mM) to culture media reduces H_2O_2-induced apoptosis and ROS production in a neuroblastoma cell line (94). Pyruvate also reduces ROS formation due to ischemia–reperfusion injury in perfused guinea pig hearts (95). Rates of glycolysis play an important role in regulating intracellular pyruvate levels, as increasing the glucose concentration of cell culture media from 2.5 to 12 mM increases intracellular pyruvate levels >10-fold in a pancreatic β-cell line (96).

The relatively low rate of glycolysis in auto- and allo-reactive lymphocytes suggests that these cells might have depleted antioxidant levels. Indeed, T cells from patients with lupus have 30% less reduced glutathione than cells from healthy controls (91), whereas T cells from MRL/lpr mice with lupus have fourfold less reduced glutathione than controls (97). Similarly, GVHD-causing T cells have 25% less total glutathione and more than 10-fold less pyruvate than control cells, consistent with their reliance on OXPHOS rather than aerobic glycolysis (67). Proliferating cells utilize NADPH for biomass synthesis to facilitate proliferation as well for reduced glutathione production. The depletion of glutathione in proliferating GVHD-causing T cells suggests that these cells might prioritize the ability to proliferate over the ability to protect themselves against oxidative insult and allocate NADPH accordingly; however, the mechanisms controlling any such NADPH allocation remain unknown.

In contrast to these results, lymphocytes activated *in vitro* increase antioxidant levels, consistent with their high rates of glycolysis. PBMCs stimulated with mitogens for 2 days double their glutathione levels compared with unstimulated cells (98), and acutely stimulated thymocytes increase glucose-derived pyruvate by 14-fold (13). Although there is no data on antioxidant levels in *in vivo* models of acute T-cell activation, the high rates of glycolysis observed in T cells activated by SEB suggests that these cells will avoid antioxidant depletion (14). These data suggest that unlike chronically activated disease-causing lymphocytes, T cells activated in an acute setting maintain their antioxidant levels, which suggests that acutely activated lymphocytes will be better able to survive an oxidative insult than disease-causing cells. Given the role that ROS play in mediating T-cell contraction and apoptosis (99), antioxidant depletion in disease-causing lymphocytes may help limit disease severity and suggests that efforts to treat autoimmune diseases or GVHD with antioxidants may be less successful than anticipated (92).

Modulating T-cell metabolism as a therapeutic strategy

If activated T cells uniformly utilized a single metabolic pathway (e.g. aerobic glycolysis) (Fig. 1), it would be difficult to exploit this pathway for therapeutic purposes. Inhibiting glycolysis might not only improve autoimmune and inflammatory diseases, but also lead to widespread immunosuppression. Similarly, augmenting lymphocyte glycolysis might aid in Treg generation and improve immunization responses but also promote autoimmunity and inflammation. Fortunately, individual lymphocyte subsets differentially utilize metabolic pathways, making activated lymphocyte metabolism a promising target for selective immunomodulation.

Activated Th17 T cells preferentially upregulate glycolysis and the glycolytic inhibitor 2DG decreases IL-17 production and disease severity in EAE (46). While inhibiting glycolysis is a promising immunomodulatory strategy and likely spares FAO-dependent Treg and memory CD8^{+} cells, there are several concerns about undesired side effects. The high rate of glycolysis observed in CD8^{+} Teff cells and acutely activated CD4^{+} T cells suggests that the inhibition of glycolysis might be associated with non-specific immunosuppression. Similarly, agents that inhibit glycolysis might decrease hematopoietic differentiation and the reconstitution of the hematopoietic system in the setting of bone marrow transplant, given the high glycolytic activity of hematopoietic stem cells (HSCs) and proliferating bone marrow cells (67, 100).

The T cells that cause GVHD and the bulk population of lymphocytes investigated in lupus share several metabolic features including increased OXPHOS and depleted antioxidants. Like a reliance on glycolysis or FAO, these metabolic features are potential targets for therapeutic manipulation. Furthermore, the ability of normal lymphocytes to maintain their antioxidant levels suggests that such a strategy will cause minimal immunosuppression.

Bz-423 is a non-anxiolytic 1,4-benzodiazepine that targets the oligomycin-sensitivity conferring protein subunit of the
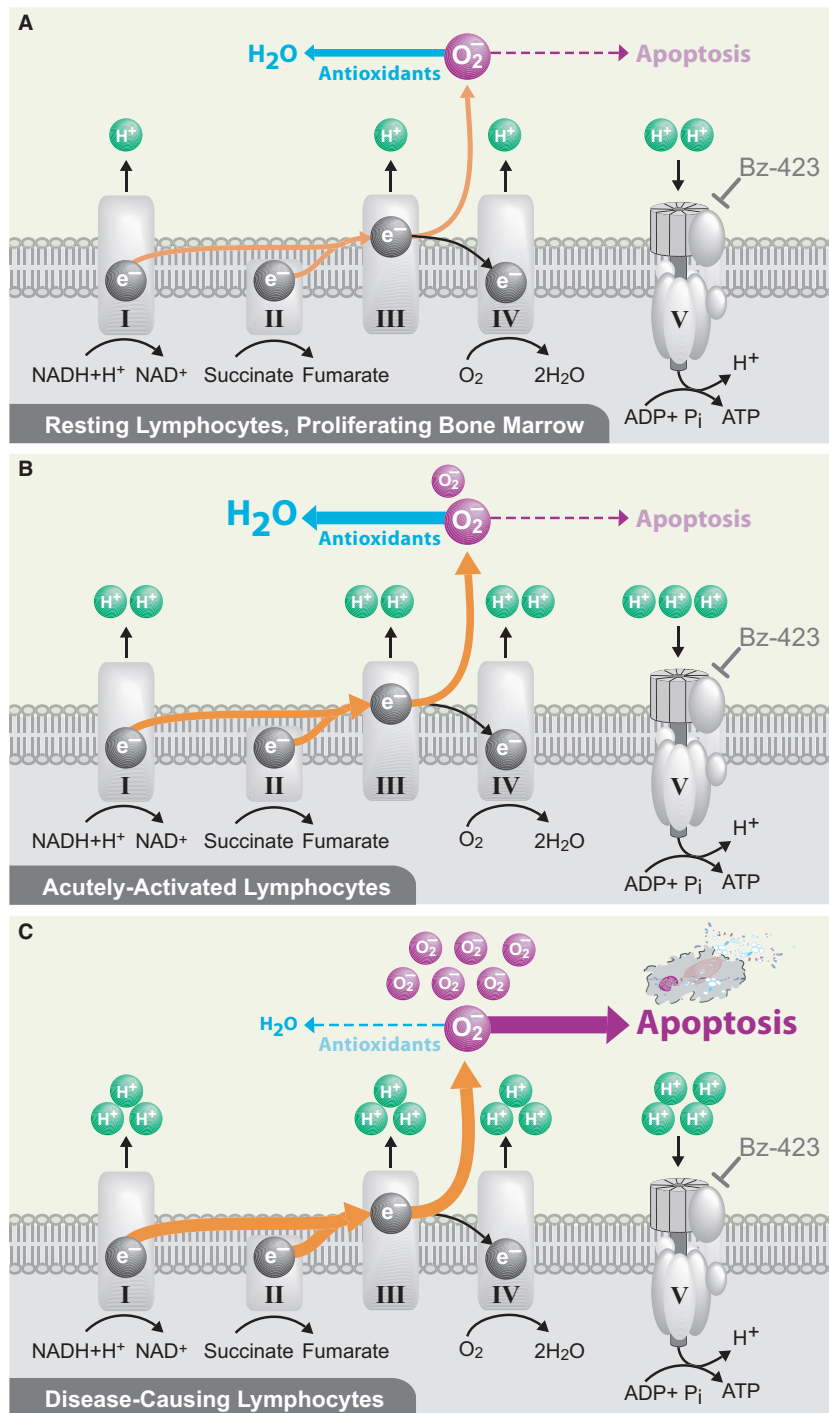
Fig. 4. Mechanism for selective induction of apoptosis in disease-causing lymphocytes by Bz-423. In the mitochondrial electron transport chain (ETC), complexes I and II accept electrons from NADH and succinate and pass these electrons (gray) to complexes III and IV, where they are eventually transferred to O_2 and form H_2O. This process pumps protons (green) into the intermembrane space, and these protons flow through complex V to generate ATP. Electrons can escape from the chain at several sites where they directly react with O_2 and generate O_2^- (purple). Once formed, O_2^- can either be detoxified by antioxidants or induce oxidative damage and apoptosis. Bz-423 inhibits complex V and leads to proton accumulation in the intermembrane space (hyperpolarization), which causes increased electron escape from the ETC and O_2^- production. The effects of Bz-423 increase as rates of oxidative phosphorylation (OXPHOS) increase. (A) Resting lymphocytes and proliferating cells in the bone marrow have low rates of OXPHOS, and thus little O_2^- is produced by Bz-423. The little O_2^- produced is efficiently detoxified by the basal levels of antioxidants present in these cells and apoptosis is avoided. (B) Acutely activated lymphocytes have modestly increased rates of OXPHOS and thus produce a modest amount of O_2^- in response to Bz-423. However, the high levels of antioxidants in these cells effectively detoxify the O_2^- formed, and apoptosis is avoided. (C) Disease-causing lymphocytes have high rates of OXPHOS and produce large amounts of O_2^- in response to Bz-423. This O_2^- cannot be detoxified due to antioxidant depletion and instead induces apoptosis.

F_1F_0-ATPase (101). Bz-423 slows the flow of protons through the F_1F_0-ATPase inducing a state 3-to-state 4 respiratory transition that leads to hyperpolarization of $\Delta\psi_m$, O_2^- production, and apoptosis (101–104) (Fig. 4). Consistent with its mechanism, Bz-423 preferentially produces ROS when mitochondria are actively respiring, whereas cellular antioxidants protect against Bz-423-mediated O_2^- production and apoptosis (102, 103, 105, 106). As auto- and allo-reactive lymphocytes have high levels of respiration and depleted antioxidants, Bz-423 induces apoptosis in these cells resulting in significant disease improvement in models of lupus and GVHD (67, 102, 107) (Fig. 4). Importantly, Bz-423 does not broadly induce lymphocyte apoptosis, affect the number of T cells in treated mice, or alter the normal immune response to challenge with foreign antigen. This selectivity differentiates this class of immunomodulators from agents used in the clinic or that are under development.

The properties of Bz-423 have recently been studied in several murine models of GVHD. Following bone marrow transplant, two distinct sets of proliferating hematopoietic cells coexist: Alloreactive donor T cells responding against host antigens and HSCs and other progenitors repopulating the host immune system. Although GVHD-causing T cells upregulate OXPHOS and have low levels of antioxidants, proliferating bone marrow cells upregulate glycolysis, have low levels of OXPHOS, and maintain their antioxidant levels (67). Treatment with Bz-423 increased ROS production and apoptosis in GVHD-causing T cells but did not affect proliferating bone marrow cells or bystander lymphocytes. Consistent with these results, Bz-423 rescued mice from lethal GVHD but did not adversely affect the repopulation of donor thymocytes, granulocytes, or lymphocytes (67).

Concluding remarks

Although it has been known for the past several decades that acutely activated lymphocytes generate their ATP using the Warburg effect, particularly in vitro, emerging evidence demonstrates that different lymphocyte subsets meet their metabolic demands by differentially utilizing fundamental metabolic pathways. The bioenergetic profile adopted by an individual T cell appears to depend on a number of factors including the chronicity of activation, the availability of nutrients, and the cytokine environment in which a T cell differentiates. On a molecular level, the regulation of T-cell metabolism involves a network of molecular regulators including AKT, HIF-1α, ERR-α, and Myc.

Recent studies have found that the metabolic phenotype adopted by a particular T-cell subset facilitates its function. The Myc-directed activation of glycolysis and glutaminolysis facilitates biomass synthesis and proliferation early during the activation of $CD4^+$ and $CD8^+$ T cells, whereas HIF-1α-mediated glycolysis likely facilitates the hypoxic survival of Th17 cells. By contrast, the FAO favored by Tregs and memory $CD8^+$ T cells likely allows these cells to survive when growth factors and glucose uptake have become limiting and emphasizes that these cell types must survive long term in the absence of proliferative signals to provide long-term immunity and control inappropriate immune responses. In the setting of GVHD, disease-causing T cells generate ATP through OXPHOS and may use their limited glucose supply to fuel proliferation rather than produce antioxidants, which leaves them vulnerable to redox-induced apoptosis. The different metabolic phenotypes adopted by activated T-cell subsets not only teach us about the function and biology of these subsets but also provide the opportunity for the therapeutic manipulation of activated T-cell metabolism.

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