T cell metabolism in graft-versus-host disease

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Abstract
Graft-versus-host disease (GVHD) is a major source of morbidity and mortality following allogeneic hematopoietic stem cell transplant (allo-HSCT), one of the most effective approaches to treat hematopoietic malignancies.1 However, current prophylaxis regimens and treatments that reduce the detrimental effect of acute GVHD can be offset by increased incidence in opportunistic infections and relapse of the primary malignancy.2 In addition, the majority of the approaches that inhibit T cell responses are non-specific, resulting in the inhibition of both alloreactive T cells and protective T cells from the donor. Therefore, there is an increase in the demand to develop novel approaches that selectively target alloreactive T cells. One potential means to address this issue is to take advantage of the unique metabolic profile of activated T cells.

Keywords: Graft-versus-host-disease, Metabolism, T cells

1. INTRODUCTION
Graft-versus-host disease (GVHD) occurs when immune responses are directed against foreign antigens. Patients whose immune system or failing organs that need replacement from donors are at risk for GVHD, given that the donor and the recipient are genetically non-identical. In those with aggressive hematologic malignancies or immunodeficiencies, hematopoietic stem cell transplant (HSCT) from an allogeneic donor can be performed as a curative option. However, GVHD accounts for a major source of morbidity and mortality aside from relapse of the primary disease.1

T cells, as part of the adaptive immunity, are one of the primary causes for the development of GVHD.3 In Major histocompatibility (MHC)-mismatched donor-recipient pairs, allogeneic T cells are activated upon recognition of the alloantigen presented by the mismatched-MHC molecule, causing damage in target tissues.4 Such reactions can also be mediated by minor histocompatibility antigens (MiHAs), which arise from differences in single nucleotide polymorphisms (SNPs) among individuals.5 In HSCTs, recipient antigen presenting cells (APCs) activate donor T cells to elicit damage in target organs.3

Therapies including T cell depletion from the donor bone marrow have been used in the clinic to reduce the risk of GVHD.5,6 However, this procedure also offsets the graft-versus-tumor (GVT) effect that is required to prevent relapse of the primary disease, particularly in the case of allogeneic HSCT.7 Since activated alloreactive T cells exhibit a unique fine-tuning profile of metabolic pathways, such signatures can be utilized to improve the treatment of GVHD by using a T cell-specific targeting approach.

2. T CELL METABOLISM
In the resting phase, T cells remain naive and primarily depend on oxidative phosphorylation (OXPHOS) to sustain survival and trafficking.8 Compared to aerobic glycolysis, OXPHOS prioritizes energy conservation, which produces 36 Adenosine triphosphate (ATP) molecules in contrast to 2 ATP molecules.8,10 This mode of metabolism best matches the functional demands of a resting T cell. Although migration through circulation, including secondary lymphoid organs, can be an ATP-exhausting process, immune surveillance is required for resting naïve T cells to screen for foreign antigens prior to activation, hence the requirement for efficient energy production.8

T cell priming occurs with T cell receptor (TCR) ligation and stimulation of the costimulatory molecules by APCs. Upon priming, T cell metabolism is fundamentally reprogrammed to adapt to the energetic demands of an activated T cell. Rather than predominately relying on OXPHOS, T cells rapidly increase the rate of aerobic glycolysis.11 Although OXPHOS is much more efficient in ATP generation, glycolysis provides various intermediate metabolites for nucleotide and amino acid production to support cell growth and division.8,11

Early T cell activation occurs from minutes to hours, and is largely independent of transcription and translation.12 Therefore, although increased aerobic glycolysis is initiated during this phase, glucose uptake and glycolytic enzymes are not yet affected. Rather, during this process, pyruvate dehydrogenase kinase (PDK1) is activated via TCR ligation to redirect pyruvate to lactate production rather than entering the tricarboxylic acid cycle.
(TCA) cycle.\textsuperscript{12} Essentially, PDHK1 acts as a metabolic switch that determines the fate of pyruvate by deactivating pyruvate dehydrogenase through phosphorylation, blocking the conversion of pyruvate into acetyl-CoA. Therefore, PDHK1 activation directly ensures that pyruvate, a metabolic intermediate of glucose, is directed toward aerobic glycolysis.

During late T cell activation, which can take hours to days, various glycolytic enzymes are upregulated to maximize aerobic glycolysis. This second spike of aerobic glycolysis is associated with transcriptional reprogramming in a Myc-dependent manner process.\textsuperscript{13} In addition, Akt (also known as protein kinase B) and hypoxia-inducible factor 1 (HIF-1α) activation also contribute to this process.\textsuperscript{14} Glucose transporters, primarily Glut1, import extracellular glucose to keep up with the increased demand for glycolysis.\textsuperscript{15} During activation, surface expression of glucose transporters is rapidly increased following Akt activation.\textsuperscript{16} Late during the T cell activation phase is also accompanied by the clonal expansion phase, during which T cells undergo rapid division. This phase requires the efficient engagement of both glycolysis and OXPHOS. Several metabolites derived from the TCA cycle can be used for de novo synthesis component required for cell growth. Among these metabolites, citrate and oxaloacetate can be used to fuel lipid and nucleotide synthesis, respectively.\textsuperscript{16,17}

During the effector phase, aerobic glycolysis continues to play a critical role. The increase in effector functions can be achieved through the overall upregulation of translation as directed by mammalian target of rapamycin complex 1 (mTORC1) activation.\textsuperscript{18–20} Not only does aerobic glycolysis meet the demand for growth and rapid cell division, increased biosynthesis also supports the production of effector molecules, including proinflammatory cytokines, IFN-γ, and cytotoxic molecules such as TNF-α and perforin.\textsuperscript{21} Overall, the increased output of the proinflammatory molecules can initiate and sustain the damage of alloreactive T cells on recipient tissues. In addition, many glycolytic enzymes act as a switch to control effector cytokine production. When glycolysis is rapidly upregulated, the translation of many cytokines become activated due to alleviated suppression by corresponding glycolytic enzymes.\textsuperscript{21} Prior studies demonstrated that T cell effector function, primarily cytokine production, is regulated by aerobic glycolysis through posttranscriptional changes. Specifically, these studies showed that many glycolytic enzymes are linked to cytokine production by binding to AU-rich elements (AREs).\textsuperscript{12,21} When not actively engaged in glycolysis (resting T cells), glycolytic enzymes bind to AREs located in the 3′UTR of cytokine mRNAs, blocking their translation. For example, Glycereraldehyde-3-Phosphate Dehydrogenase (GAPDH) represses Ifng translation when the glycolytic flux is low.\textsuperscript{22} Besides regulating Ifng, the lactic acid dehydrogenase (LDH) can also control the translation of Tnfα, Il2, limiting the synthesis of proinflammatory cytokines.\textsuperscript{12} However, cytotoxic granules including perforin and granzyme B lack the ARE component, and are not under the control of glycolytic enzymes like cytokines.\textsuperscript{22} Nevertheless, the translational process is tightly coordinated with aerobic glycolysis through mTORC1 activation, indicating that cytotoxic granules are controlled through translation.

In terms of differences among distinct T cell lineages, CD4\textsuperscript{+} T cell subsets also display unique signatures of metabolic requirements. Though differences exist, Th1, Th2, and Th17 cells preferentially utilize aerobic glycolysis.\textsuperscript{8,23,24} In contrast, regulatory T cells (Tregs) favor fatty acid oxidation (FAO).\textsuperscript{25,26} Manipulation of metabolic pathways can also influence T helper cell differentiation.\textsuperscript{8} The addition of lipids favors the generation of Tregs, rather than other effector lineages.\textsuperscript{27} Similarly, memory T cells predominately rely on FAO,\textsuperscript{28,29} accompanied by increased expression of the lipid transporter, carnitine palmitoyltransferase 1A (CPT1A), located on the mitochondrial membrane.\textsuperscript{30,31} Other relevant metabolic reprogramming of memory T cells include increased mitochondrial biomass and spare respiratory capacity, conferring greater resistance to metabolic stress.\textsuperscript{1,28} These metabolic changes are critical to ensure memory T cell survival following an immune response.

Similar to CD4\textsuperscript{+} T cells, the activation of CD8\textsuperscript{+} T cells into cytotoxic effector T cells also requires the upregulation of aerobic glycolysis to keep up with the biosynthetic demands.\textsuperscript{13} Both rapid division and production of proinflammatory cytokines, as well as cytolytic granules are highly dependent on this process.

\section*{3. T CELL METABOLISM DURING GVHD: THE ROLE OF DIFFERENT METABOLIC PATHWAYS IN ALLOREACTIVE T CELLS}

\subsection*{3.1. Aerobic glycolysis}

Aerobic glycolysis is classically defined in the Warburg effect as the conversion of pyruvate into lactate as opposed to being utilized for TCA cycle even in the presence of sufficient oxygen.\textsuperscript{3,32,33} Though a concept familiar to cancer studies as this pathway is highly upregulated in many types of tumor cells, it is now becoming increasingly clear that activated lymphocytes, including T cells, also rely on aerobic glycolysis for rapid biosynthesis and proliferation.\textsuperscript{15,34}

Previous studies showed that aerobic glycolysis supports T cell growth and proliferation.\textsuperscript{15,35} Although this process is less efficient at generating ATP compared to OXPHOS, the various intermediate metabolites generated through this pathway support cell growth and division. Immediately following TCR ligation, aerobic glycolysis is rapidly initiated through PDHK1, which allows for the conversion of the end product of glycolysis, pyruvate, into lactate rather than feeding the TCA cycle. Subsequently, costimulatory molecule ligation by the APC (CD28) activates the PI3K-Akt-mTOR signaling pathway.\textsuperscript{36} Akt can upregulate glycolysis by phosphorylating glycolytic enzymes such as hexokinase (HK) to increase the glycolytic flux.\textsuperscript{3,17} In alloreactive T cells, metabolically, the expression of a second isoform, HK2, is also enhanced to drastically increase the rate of glycolysis.\textsuperscript{37} Akt is also critical for the surface trafficking of Glut1 to achieve increased glucose uptake.\textsuperscript{38} Downstream of Akt, the mTORC1 is activated and promotes translational efficiency through the phosphorylation of eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and p70S6 kinase (p70S6K).\textsuperscript{39}

We and other have shown that alloreactive T cells preferentially upregulate glycolysis when activated by alloantigens (Fig. 1).\textsuperscript{1,37} This was demonstrated by increased glycolytic activity in T cells isolated from transplant recipients, as well as the requirement for Glut1 to mediate GVHD development in a murine bone marrow transplant model. Compared to syngeneic transplants, in which T cells also upregulate glycolysis during homeostatic expansion in response to cytokines or self-ligands, allogenic transplants differ in that alloreactive T cells upregulate glycolysis much higher than the syngeneic counterparts. In addition, aside from the upregulation of Glut1 by alloreactive T cells, Glut3 expression is also increased to further enhance glycolysis compared to T cells derived from syngeneic recipients.\textsuperscript{37}
In terms of the role of aerobic glycolysis in CD4+ T cell subset differentiation and function, Th1, Th2, Th17 are pathogenic in the context of GVHD and prefer the use of glycolysis.40 In contrast, Tregs are suppressive and prevent the progression of target organ.47 This provides implications for the impact of glycolytic inhibition on GVHD versus GVT. Since the GVT effect is primarily mediated by cytotoxic donor T cells recognizing the tumor antigen, it would be beneficial for CD8+ tumor-specific T cells to survive from glycolytic inhibition.

### 3.2. OXPHOS

As a critical energy-conserving component of the catabolic pathway, OXPHOS is a tightly regulated process that allows lymphocytes to adapt to metabolic stress and changes in cellular needs. OXPHOS is tied to the regulation of aerobic glycolysis due to the competition for pyruvate availability. Since aerobic glycolysis is under the control of the energy sensor adenosine monophosphate-activated protein kinase (AMPK), the activity of AMPK can regulate mitochondrial oxidative capacity via OXPHOS.48–50 In resting T cells and memory T cells, AMPK-mediated oxidative metabolic state promote cell survival and help them adapt to the corresponding energetic needs.8,51

In activated T cells, AMPK activation is triggered immediately following T cell activation due to increased LKB1 signaling and escalated intracellular calcium level, which is a transient process.52,53 This is followed by the activation of mTORC1, which is preceded by the inhibition of AMPK.48 Therefore, activated T cells have lower AMPK activity and higher glycolytic rate to support growth and effector functions. These findings have implications in the setting of an inflammatory response. Indeed, CD8+ T cells deficient in AMPKα become more potent proinflammatory cytokine producers.53 Previous studies using murine allo-HSCT models suggest that OXPHOS is actively utilized at comparable levels in both syngeneic and allogeneic BMT in mouse studies.37 In addition, lower levels of TCA cycle metabolites such as citrate, fumarate, and malate were found in alloreactive T cells compared to T cells derived from syngeneic BMT in mouse studies.37 This result points to the possibility that pyruvate molecules were predominately converted to lactate.
rather than TCA intermediates, highlighting a dominant role for aerobic glycolysis instead of OXPHOS during GVHD development. Interestingly, ROS production as a result of increased OXPHOS has been shown to be required for T cell activation.\(^2\) It is possible that this mechanism is also utilized by activated alloreactive T cells. Hence, it may be optimal to simultaneously inhibit aerobic glycolysis and OXPHOS, despite a more dominant role for glycolysis.

### 3.3. Lipid metabolism

During T cell activation, Myc not only mediates transcriptional changes in glucose metabolism, but also regulates genes for fatty acid synthesis (FAS).\(^3\) Moreover, lipid metabolism has been shown to regulate T cell fate.\(^5\) It was demonstrated that enhanced lipid synthesis promotes the proinflammatory effector T cell phenotype while lipid oxidation favors iTreg differentiation.\(^2\)

Previous studies using murine models demonstrated that alloreactive T cells not only displayed a tendency for the accumulation of long-chain fatty acids, but also upregulated enzymes associated with FAS, indicating that FAS may be able to promote GVHD development.\(^5\) In line with this hypothesis, a separate murine study showed that inhibition of FAS by interfering with acetyl-CoA carboxylase 1 (TACC1) prevented clonal expansion of alloreactive T cells in vitro.\(^7\) Furthermore, transfer of treated donor T cells was able to arrest the development of GVHD. Collectively, these findings indicate that the regulation of FAS, a component of anabolic metabolism similar to glycolysis, may be useful to inhibit the pathogenicity of alloreactive T cells.

As the catabolic branch of lipid metabolism, there are also studies testing the role of FAO. However, results from different groups appeared to report conflicting findings about the role of FAO in GVHD.\(^7\),\(^26\) Alloreactive T cells have been shown to display increased FAO by Ferrara’s group.\(^8\) However, other studies appeared to suggest that FAO plays a less important role, as indicated by the decreased amount of key metabolites required for FAO and TCA cycle in T cells derived from allogeneic HSCT recipients compared to syngeneic recipients in murine models.\(^3\) In addition, fatty acid uptake was also found to be lower in alloreactive T cells, contrary to the former reports. Factors contributing to discrepancies between these reports include the varying use of controls. In the first study, resting cells were used as control while donor T cells derived from syngeneic HSCT were used for the second study. Syngeneic donor T cells may be a more appropriate control in GVHD models as it accounts for background signal contributed by homeostatic proliferation.\(^8\)

### 3.4. Glutamine metabolism

Glutamine can be used by activated T cells as an alternative carbon source for TCA cycle.\(^4\) The process begins through the conversion of glutamine to glutamate. Eventually, a-ketoglutarate (a-KG), a citrate precursor, is generated via glutaminolysis.\(^4\),\(^5\),\(^6\) In addition to replenishing metabolites in TCA cycle, glutamine can also be used as a source for anabolic pathways to support cell growth.\(^9\) The production of a-KG can be used to generate citrate, which forms the backbone during lipidsynthesis once converted to acetyl-CoA in the cytosol.\(^2\) In addition, glutamine can also be used for nucleotide synthesis. Specifically, during activation of alloreactive T cells, both CD4\(^+\) and CD8\(^+\) T cell subsets utilize as substrates for ribose synthesis, promoting DNA replication during proliferation. Another facet of glutamine metabolism in GVHD is the upregulation of glutamine transporters expressions in alloreactive T cells. In particular, the expression of glutamine transporters, including SLC3a2 and SLC5A1, is controlled by Myc.\(^6\) Interestingly, Myc-regulated GLS1 expression further promotes the conversion of glutamine to glutamate.\(^6\),\(^5\)

### 3.5. Pentose phosphate pathway

The pentose phosphate pathway (PPP) is another component of anabolic metabolism, and is preferentially utilized by alloreactive T cells to promote cell growth and proliferation.\(^3\) PPP generates carbon donors (ribose-5-phosphate) for nucleotide synthesis.\(^6\) In alloreactive T cells, the glycolysis intermediate, glucose-6-phosphate (G-6-P), is used as the main substrate for PPP to produce the end product and fuel nucleotide generation.\(^6\),\(^7\) Therefore, both PPP and glycolysis activities are enhanced in alloreactive T cells. In addition, PPP also produces NADPH to support the synthesis of antioxidants,\(^8\) potentially alleviating the oxidative stress during T cell activation.

### 4. TARGETING ALLOREACTIVE T CELL METABOLISM WHILE PRESERVING GVT EFFECT

Activated alloreactive T cells display distinct metabolic signatures to promote their survival, clonal expansion, and proinflammatory effector functions during GVHD development. However, the use of broad immunosuppressant drugs, such as glucocorticoids and calcineurin inhibitors, not only can lead to many complications, but also suppresses both alloreactive and antitumor T cells, thus unable to separate GVHD targeting and the GVT effect. Therefore, studies characterizing the metabolic signatures of alloreactive T cells provide key insights for the development of drugs that will improve the specific inhibition of alloreactive T cells.

For GVHD-targeting, blockade of aerobic glycolysis has shown efficacy for alleviating disease development in murine studies. We have also demonstrated using a murine model for allo-HSCT that the deletion of Glut1 significantly alleviates GVHD development by impairing glycolysis.\(^1\) However, targeting glycolysis with small molecule inhibitors such as 2DG (interfering the HK step of glycolysis),\(^3\) 3-(3-pyridinyl)-1-propen-1-one (3-PO) (inhibiting PFKFB3, a regulatory and a rate-limiting factor in the glycolytic pathway),\(^3\),\(^27\) and rapamycin (inhibiting mTORC1 activation) may be more practical as a treatment regimen in the clinic.\(^72\),\(^74\) This approach may be promising as murine alloreactive T cells have been shown to be susceptible to glycolysis inhibitors in vitro. However, such procedures must be developed with caution to decrease the off-target effects of non-specific targeting if given systemically. Other modulators, such as programmed death 1 (PD-1), have been shown to modulate glucose metabolism in T cells. Ligation of PD-1 reduces the capacity to engage in glycolysis, causing pre-activated T cells to switch the glycolytic program in favor of FAO.\(^2\) Thus, PD-1 ligation has been demonstrated in murine studies, which showed rapid increase in mortality in PD-L1-deficient hosts, compared to wild type recipients with upregulation of PD-L1 during GVHD.\(^7\) On the other hand, AMPK activators, including metformin and AICAR,\(^7\) can be used to suppress aerobic glycolysis in activated T cells by modulating mTORC1. Furthermore, pharmacological activation of AMPK or mTORC1 inhibition may have the potential to promote iTreg polarization while preventing the generation of...
pathogenic Th17 cells, as it has been shown in vitro. Another advantage of glycolytic inhibitors is that it has the potential to separate GVHD from the GVT effect, given that tumor-specific T cells, which are CD8+ T cells that mediate direct killing, may be more resistant to metabolic inhibition than their CD4+ counterpart. Although both CD4+ and CD8+ T cells upregulate glucose metabolism upon activation, the metabolism of CD8+ T cells is more glycolytic while capable of better utilizing glutamine as an alternate energy source, and was demonstrated to be more tolerant to glycolytic inhibition in in vitro assays using murine T cells. In addition, memory CD8+ T cells that mediate GVT preferentially depend on FAO, as opposed glycolysis. Past studies have indicated that the use of glycolytic inhibitors can further enhance the antitumor activity of CD8+ memory T cells, possibly due to increased FAO to compensate for the lack of energy derivation from glycolysis.

Although alloreactive T cells primarily rely on aerobic glycolysis, OXPHOS is also increased, with ROS as a byproduct to support T cell activation. Consequently, alloreactive T cells exhibit an oxidative phenotype and are more susceptible to supernoxide-induced apoptosis. In murine allo-HSCT studies, the use of Bz-423 (induces the generation of superoxide by inhibiting mitochondrial FI50 ATP synthase) alleviated GVHD. Therefore, it is tempting to target multiple metabolic pathways simultaneously, such as OXPHOS and aerobic glycolysis, in the clinic.

Inhibitors of lipid metabolism, including etomoxir (suppression of FAO by inhibition of CPT1), can also be used to treat GVHD in the allo-HSCT setting. Etoricoxib, which inhibits CPT1, is a potential option to prevent chronic rejection. However, a concern with this approach is that iTregs differentiation may be blocked, as they are also dependent on FAO.

In order to minimize off-target effects and preserve the GV effect, ex vivo treatment of donor T cells prior to the transplant may deliver a much more precise inhibition. Donor T cells, a heterogeneous pool that contains T cells specific for pathogens, tumor antigens, and alloantigens, can be subjected to ex vivo activation with recipient alloantigens in the presence of metabolic inhibitors such as 2DG. Such treatment would potentially lead to cell death or anergy of activated alloreactive T cells. By contrast, the viability and function of beneficial T cells would be preserved since they cannot react to alloantigens and are less susceptible to glycolytic inhibition during the ex vivo suppression treatment.

5. CONCLUDING REMARKS

The targeting of metabolic pathways utilized by alloreactive T cells have demonstrated promising results in murine models, with increased survival of recipients and reduction in GVHD pathology, as well as decreased incidences of complications due to opportunistic infections. Compared to broadly immunosuppressive regimens currently available in the clinic, the use of metabolic signatures appears as a unique and promising strategy to prevent the development and progression of GVHD. Future studies should also consider the delivery of pharmacological inhibitors in a T cell-specific manner, which will reduce complications caused by systemic administration.

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