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Pharmacological effects on anaplerotic pathways alter the metabolic landscape in the tumor microenvironment, causing unpredictable, sustained anti-tumor immunity

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Abstract

To achieve sustained anti-tumor immunity, tumor-infiltrating effector CD8 T lymphocytes (CD8 TILs) must be able to produce cytokines, including IFNγ, and proliferate robustly within the local tumor tissue upon antigen recognition. IFNγ production by CD8 TILs depends on glycolysis, whereas their proliferation additionally requires oxidative phosphorylation (OxPhos). The level of OxPhos, and hence the oxygen consumption rate, depends on mitochondrial biogenesis and requires the loading of metabolic precursors into the tricarboxylic acid cycle to keep it functioning. This is referred to as anaplerosis. Recent advances in the field of immuno-metabolism have shown the impact of pharmacological agents on anaplerotic pathways, resulting in metabolic down-regulation in tumor cells; in contrast, the agents trigger sustained anti-tumor immunity by up-regulating both glycolysis and OxPhos in CD8 TILs. The opposing effects of pharmacological inhibition (and/or activation) on anaplerosis in tumor cells and CD8 TILs are unpredictable. Careful dissection of the underlying mechanism might confer important knowledge, helping us to step into a new era for cancer immunotherapy.

Keywords: immuno-metabolism, tumor immunity

Introduction

Tumor cells and T cells share many nutrients, like glucose and glutamine, for their survival and vigorous proliferation. This implies that the tumor-infiltrating effector CD8 T lymphocytes (CD8 TILs) must compete with tumor cells for these nutrients. Under conditions of effective immune surveillance, CD8 TILs can eliminate tumor cells because glycolysis is not suppressed in the effector cells. However, as per the Warburg effect, tumor cells take up large amounts of glucose and glutamine, irrespective of the oxygen concentration, which results in them limiting the function and proliferation of CD8 TILs, thereby, contributing to the generation of dysfunctional, exhausted T cells in the tumor microenvironment (TME) (1, 2). A balance between effector cells and tumor cells is referred to as the equilibrium state.

In addition to nutrient consumption, rapid growth of tumors not only decreases the pH, because of lactate production, but also lowers the oxygen concentration in the TME, which results in the accumulation of regulatory T (Treg) cells (3), myeloid-derived suppressor cells (MDSCs) (4) and tumor-associated macrophages (TAMs) (5)—the negative regulators of CD8 TILs (6–8). Low pH and a low oxygen concentration (9) directly impair the function of CD8 TILs, allowing for the progression of tumors. In this phase of immune evasion, the metabolism of CD8 TILs is skewed into the oxidative phosphorylation (OxPhos)-dominant state, following incorporation of fatty acids instead of glucose. The metabolic shift from glycolysis to OxPhos results in the conversion of CD8 TILs from the effector memory T (TEM) to the central memory T (TCM) phenotype, which is less effective in terms of fighting tumor cells.

Tumor cells attain metabolic heterogeneity during their expansion and some of them start to utilize lactate to fuel the tricarboxylic acid (TCA) cycle, followed by the elevation of OxPhos. Tumor cells that have elevated OxPhos have recently been shown to spread to distal organs (10–12). The expansion of the primary tumor and its spreading could thus be explained, at least in part, as being a result of metabolic competition between tumor cells and effector T cells (Fig. 1).

Improvement of metabolism in the TME, therefore, potentially impacts the fates of tumor cells and CD8 TILs.

Anaplerosis is defined as an enzymatic reaction that produces metabolic intermediates. The term consists of two Greek words, 'Ana' and 'Plerotikos', meaning 'Re' and 'Fill', respectively. The TCA cycle in the mitochondria is representative of an anaplerotic reaction. TCA cycle-derived metabolites are utilized as substrates for anabolic biosynthesis, such as fatty acid synthesis and gluconeogenesis, by a process referred to as cataplerosis. In contrast, anaplerosis is a process to replenish (re-fill) precursor intermediates in the TCA cycle to keep the TCA cycle functioning, in addition to OxPhos in the mitochondria (13).

The main anaplerotic pathways include production of oxaloacetate from pyruvate, phosphoenolpyruvate (PEP) and aspartate; production of α-ketoglutarate (αKG) from glutamate (or glutamine); and production of succinyl coenzyme A (CoA) from the β-oxidation of fatty acids (FAO). All these pathways are involved in maintaining the function of the TCA cycle (Fig. 2). Stimulation of the anaplerotic pathways results in the elevation of the respiratory chain function to generate more ATP; thus, the AMP/ATP ratio decreases.

Importantly, recent studies have shown that pharmacological inhibition of some metabolites (and/or enzymes) involved in the anaplerotic pathway can change the fate of immune cells and tumor cells through differential metabolic reprogramming, which is substantially directed to the generation of sustained anti-tumor immunity. In this review, we provide an overview of the unique roles of a number of nutrients and metabolic intermediates involved in anaplerosis and discuss their roles in T-cell function/proliferation and tumor cell growth. We have focused on glucose, long-chain fatty acids, glutamine and metabolites, such as lactate and acetate, that are precursors of pyruvate and acetyl-CoA, each of which is involved in fueling the TCA cycle uniquely. In addition, pharmacological intervention to inhibit anaplerotic reactions is discussed to understand how we could improve the metabolic imbalance between tumor cells and T cells to achieve more efficient cancer immunotherapy.

Glucose

CD8 TILs can produce IFNγ in response to antigen stimulation only in the presence of glucose. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is one of the rate-limiting enzymes in glycolysis and its inhibition or the absence of glucose results in the decreased translation of IFNγ (14). PEP deficiency abrogates IFNγ production in T cells as Ca^{2+} that is released from the endoplasmic reticulum into the cytosol upon TCR stimulation rapidly declines, resulting in decreased translocation of the transcription factor NFAT into the nucleus (2). Thus, glycolysis is essential for the production of IFNγ from T cells. However, glutaminolysis is also involved in the conversion of oxaloacetate to PEP by phosphoenolpyruvate

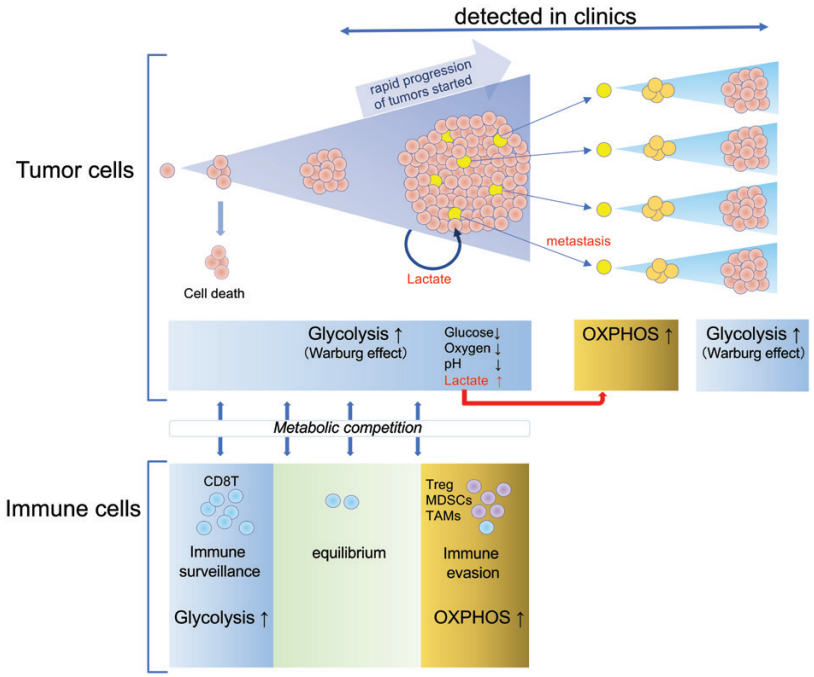

Fig. 1. Expansion of a primary tumor and its spreading to distal organs as a result of metabolic competition with immune effector cells. In the initial phase of tumor growth, CD8 TILs have the capacity to eliminate tumor cells because glycolysis is maintained at a high level, which is referred to as immune surveillance. As a result of metabolic competition with tumor cells, CD8 TILs are gradually starved of glucose and lose their functions, including production of multiple cytokines and killing of tumor cells, allowing rapid growth of the tumor. The rapid progression of the tumor provides further nutrient-stress to the TME; thus, creating an extremely low concentration of glucose and oxygen, while decreasing the pH by secreting a large amount of lactate. This forces CD8 TILs to utilize fatty acids for their survival and skews the phenotype toward TCM from TEM, while recruiting more Treg cells, MDSCs and TAMs. Nutrient-stress also gives tumor cells a pressure to acquire metabolic heterogeneity. Some tumor cells whose MCT expression is very high begin to uptake lactate, followed by the elevation of OxPhos, which enables substantial metastasis of tumor cells to distal organs.

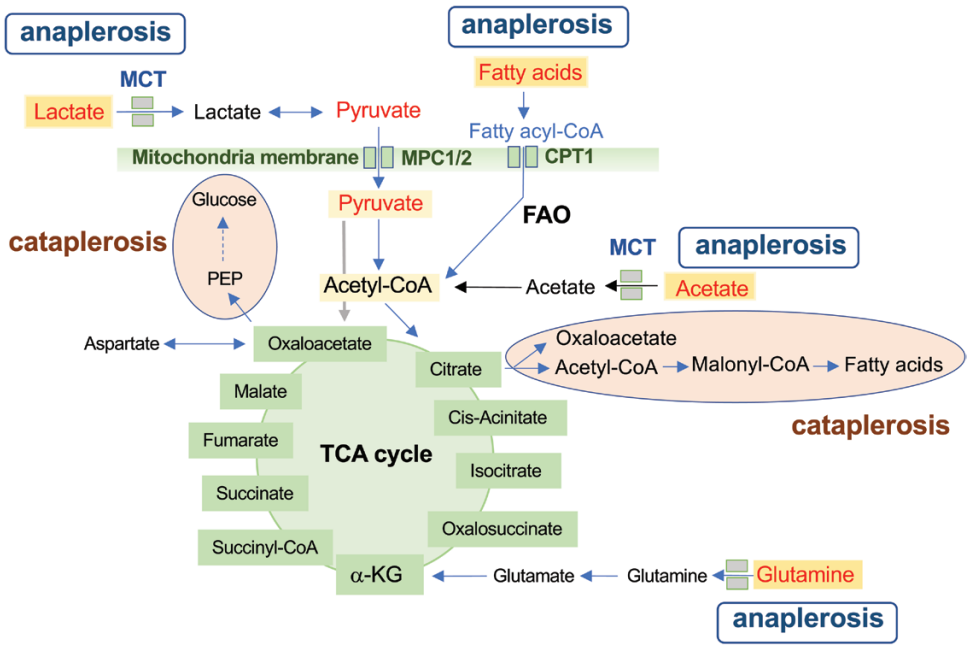

Fig. 2. Anaplerosis and cataplerosis for the citric acid cycle.

carboxy kinase 1 (PCK1), which enables the production of IFNγ in CD8 TILs (2).

Recently, studies on tertiary lymphoid structures (TLS), whose appearance in tumors correlates with a better prognosis following immune-checkpoint blockade therapy, have suggested that they could serve as a site for the activation and differentiation or proliferation of naive T cells into effector cells and the re-activation of TCM cells into TEM cells (15, 16). However, glucose availability in TLS would determine the fate of T cells, as activation of naive T cells upon triggering of the TCR under conditions of limited glucose caused the induction of anergic T cells (2). In addition to its role in maintaining effector T-cell function, glucose contributes to maintaining the function of the TCA cycle. Glucose is oxidized into pyruvate, which is further converted to acetyl-CoA and oxaloacetate by pyruvate dehydrogenase (PDH) and pyruvate carboxylase (PC), respectively (Fig. 3). Therefore, glucose is an important nutrient, converging on metabolites to fuel the TCA cycle and, hence, on anaplerosis. Lung metastasis of breast carcinoma depends on PC-mediated anaplerosis (pyruvate → oxaloacetate) and not glutamine-dependent anaplerosis (glutamine → oxaloacetate) (17). PC-dependent tumor survival and proliferation have also been reported in non-small-cell lung cancer (NSCLC) (18).

Acetate

Tumor cells can use the short-chain fatty acid, acetate, as an alternative carbon substrate to fuel the TCA cycle and cell proliferation under limited glucose conditions (19–21). Similarly, even when glucose is limited, acetate activates IFNγ production in exhausted CD8 TILs. Acetate is converted to acetyl-CoA by acyl-CoA synthetase short chain 2 (ACSS2) upon uptake via the monocarboxylate transporter (MCT); the acetyl-CoA thus produced donates an acetyl group and promotes histone acetylation and chromatin accessibility in T cells to stimulate IFNγ production (Fig. 3) (22).

Importantly, T-cell proliferation is not restored by supplementation with acetate, suggesting that acetate can only substitute for glucose in cytokine production and not during other metabolic processes like cell proliferation. This is because acetate in this case does not activate the TCA cycle. The importance of acetate in T-cell function has been described previously (23). Moreover, activation of exhausted CD8 TILs by glutamine blockade seems to require this pathway (24). Thus, acetate-dependent activation of T cells when the glucose concentration is limited, as in the TME, is important and will be discussed later in this review.

Glutamine

Glutamine is essential for rapidly dividing healthy cells and tumor cells (25–27). Glutamine is thus a potential target for cancer therapy (28, 29). Following uptake of glutamine via the amino acid transporters, glutaminase (GLS) hydrolyzes glutamine to glutamate, which is further converted to αKG to fuel the TCA cycle. αKG is also required by histone and DNA demethylases, thereby affecting chromatin accessibility (30).

Glutamine deprivation promotes the expression of Foxp3, a master transcription factor of Treg cells (31, 32), whereas GLS inhibition does not (33), implying that the effect of GLS inhibition is distinct from glutamine deficiency. GLS inhibition elevates T-bet and promotes the production of IFNγ and IL-2 from Th1 cells, whereas it reduces the levels of retinoic acid-related orphan receptor γt (RORγt) and down-regulates IL-17A production in Th17 cells. GLS-deficient CD8 T cells express more IL-2 and granzyme B upon TCR stimulation (32). Transposase-accessible chromatin sequencing (ATAC-seq) results revealed that GLS inhibition dramatically increased chromatin accessibility in Th1 cells and produced inaccessible regions

in Th17 cells (33). Furthermore, RNA-Seq analysis uncovered strong down-regulation of phosphoinositide-3-kinase (PI3K)-interacting protein 1 (PIK3IP1) by GLS inhibition. As PIK3IP1 is a negative regulator for PI3K and mammalian target of rapamycin C1 (mTORC1) in T cells, GLS inhibition causes the induction or activation of Th1 cells and cytotoxic T lymphocytes (CTLs) (33). Thus, the using inhibitors of the glutamine pathway seems to be an attractive approach for cancer treatment and will be discussed later.

Lactate

Lactate is the end-product of glucose oxidation; thus, it was thought as a 'waste product' of glycolysis and was eliminated from tumor cells (34). It is secreted from tumor cells through the MCT and decreases the pH in the TME, thereby, suppressing T-cell function. In contrast, some tumor cells can uptake lactate during *in vitro* culture (35, 36). Even *in vivo*, lung and pancreatic cancers use the MCT (which is a bidirectional, passive transporter of lactate) to transport lactate from the blood into the tumor (10, 37). The import of lactate by tumor cells contributes to the production of mitochondrial pyruvate and acetyl-CoA that can fuel the TCA cycle, stimulating the mitochondrial respiratory chain (Fig. 3). The incorporation of lactate by tumor cells and its use for anaplerosis worsens clinical outcomes (10). Thus, lactate-dependent OxPhos elevation promotes the spreading of the tumors to distant organs (11, 12). Pharmacological inhibition of OxPhos in tumor cells could be a reasonable approach for blocking metastasis from primary tumors.

Fatty acids

During tumor progression, CD8 TILs rely heavily on FAO for their metabolism because of low glucose and low oxygen levels in the TME. In fact, experiments with CD8 T cells that had been labeled with $^{13}C_6$-glucose and $^{13}C_{16}$-palmitate isotopes *in vitro* revealed that glucose-dependent carbohydrate was decreased, while fatty acid-derived carbons were increased, in the acetyl-CoA and the TCA cycle metabolites, under limited concentrations of glucose and oxygen (38). In late stages of tumor progression, CD8 TILs show elevated expression of peroxisome proliferator-activated receptor α (PPAR-α) and the FAO rate-limiting enzyme, Cpt1a (carnitine palmitoyl transferase 1a), suggesting a metabolic switch to OxPhos. In this phase, CD8 TILs lose mitochondrial membrane potential and increase the mitochondrial reactive oxygen species (ROS) level. The enhanced FAO metabolism of CD8 TILs is supported by a high abundance of free fatty acids in tumors (38).

This evidence suggest that CD8 TILs with elevated FAO and decreased glycolysis allow vigorous tumor progression. This idea is seemingly in conflict with the results seen from adoptive transfer experiments that used T cells. In the adoptive transfer experiments, antigen-specific CD8 T cells in which HIF1α (a major transcription factor regulating cells' response to hypoxia, and Glut-1 is a downstream molecule) was knocked down by short hairpin RNA (shRNA) showed better anti-tumor effects although Glut-1 expression was decreased (38). The improved anti-tumor effect was achieved by enhanced expression of PPAR-α that promotes FAO and the uptake of fatty acids. Adoptively transferred T cells must at first proliferate *in vivo* as, otherwise, they cannot be detected in the tumor by flow cytometry analysis. Cell proliferation requires anabolic metabolism for biomass production and, hence, FAO. However, the artificial activation of PPAR-α not only promotes cell proliferation but also stimulates cytokine production in CD8 T cells.

In fact, fenofibrate (FF), a PPAR-α agonist, stimulates polyfunctionality of CD8 T cells cultured under hypoglycemia and hypoxia (38). In addition to the adoptive transfer system, activation of FAO by chemicals *in vivo* facilitates mitochondrial activation in CD8 TILs to achieve a synergistic effect with PD-1 blockade (PD-1 is a target of immune-checkpoint therapy) (39, 40). Other reports also suggest that targeting FAO and OxPhos in CD8 TILs has better anti-tumor effects (41, 42), whereas some reports insist that targeting glycolysis is better (14).

This discrepancy is likely due to the experimental systems, including the nature of the tumors. For example, the presence of leptin and/or PD-1 ligation in CD8 TILs activates signal transducer and activator of transcription 3 (STAT3) to increase FAO, while decreasing glycolysis and cell functions (43). FAO driven by STAT3 in CD8 TILs plays a role in developing obesity-associated breast tumors (43). In either case, both glycolysis and OxPhos are theoretically required for the maintenance of fully activated, proliferating effector T cells in the TME.

Metabolic intervention to alter anaplerotic reaction improves anti-tumor immunity

Blocking glutamine-related pathways, including glutaminolysis, was thought to hamper T-cell function or proliferation, although tumor cell proliferation was seen to be inhibited. However, Leone *et al*. have recently shown that blockade of glutamine (but not glutaminolysis) stimulated the activation and proliferation of CD8 TILs, while keeping their phenotype as long-lived memory cells (24). This was achieved by a newly developed inhibitor, JHU083, which is a derivative of the glutamine inhibitor, 6-Diazo-5-oxo-L-norleucine (DON). Intriguingly, the metabolism (both glycolysis and OxPhos) of tumor cells (MC38) was down-regulated, as determined by the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR), respectively, *in vitro* by DON treatment. In contrast, both ECAR and OCR were up-regulated in P14 CD8 T cells by DON treatment *in vitro*. Moreover, the OCR/ECAR ratio of CD8 TILs in MC38 tumors was significantly increased by JHU083 treatment, indicating that OxPhos was dominant over glycolysis.

The effects were intrinsic to T cells but were not achieved by the blockade of MC38 cell metabolism *in vivo*, because the phenomena were recapitulated *in vitro* in P14 CD8 T cells. Detailed analysis revealed that glutamine blockade inhibits not only the TCA cycle but also glycolysis in MC38 tumor cells; in contrast, the same treatment promotes the activation of the TCA cycle and glycolysis in T cells. The differential outcome was derived from the state of enzyme activation. Activation of PC and ACSS1 was observed in CD8 TILs but not in MC38 cells by glutamine blockade. PC converts

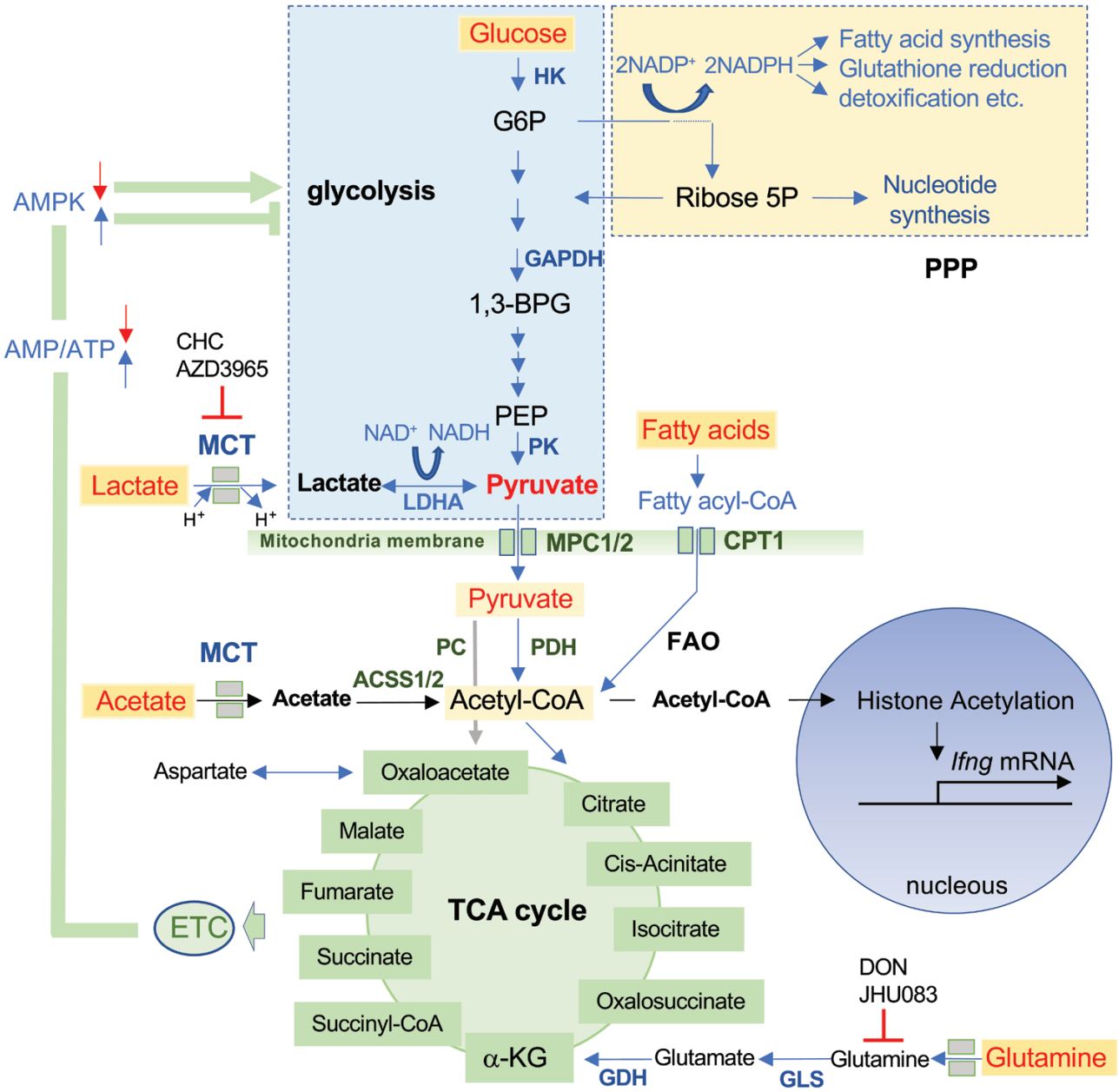

Fig. 3. Anaplerosis is involved in the regulation of both OxPhos and glycolysis. Feeding metabolites, including glucose, pyruvate, lactate, fatty acids and glutamine, into the TCA cycle (anaplerosis) elevates respiratory chain function, resulting in the production of more ATP; thus, decreasing the AMP/ATP ratio. The decreased AMP/ATP ratio down-regulates AMPK activity, which in turn activates glycolysis in CD8 TILs. Incorporation of acetate results in the production of acetyl-CoA that is utilized for histone acetylation, thereby helping in the transcription of the gene encoding IFNγ in CD8 TILs. On the other hand, incorporation of lactate via MCT in tumor cells results in the production of pyruvate and stimulation of the PPP. Loading pyruvate to the TCA cycle results in anaplerosis and thereby stimulates OxPhos, accelerating the spread of the tumor to distal organs. The driving of the PPP generates NADPH, helping eliminate H_2O_2, thereby enabling metastasis of tumor cells.

pyruvate to oxaloacetate, to fuel the TCA cycle, and ACSS1 is involved in the conversion of acetate to acetyl-CoA, contributing to histone acetylation, to stimulate the transcription of the gene encoding IFNγ. The TCA cycle promotes OxPhos to produce ATP and thereby suppress AMP-activated protein kinase (AMPK), which in turn activates glycolysis and the pentose phosphate pathway (PPP) in T cells (Fig. 3) (44, 45).

Importantly, PPP is also driven by glutamine blockade in T cells and is involved in the elimination of ROS. ROS elimination is thus essential in highly proliferative cells—both normal cells and tumor cells. Why is the Warburg effect essential in tumor cells? The driving of the PPP that emanates from glycolysis to remove ROS might be a reason for this.

Metformin (Met), one of the biguanides, is a first-line drug for type 2 diabetes mellitus (T2DM). We have previously shown that the Met-induced anti-tumor effect is mediated by tumor-specific CD8 T lymphocytes (46). Met targets complex I in the mitochondrial respiratory chain, allowing the production

of mitochondrial ROS (H_2O_2) that stimulates Glut-1 expression on the surface of CD8 TILs; thus, increasing glycolysis. The PPP is also activated to eliminate ROS, which indicates that Nrf2, a major transcription factor for the anti-oxidative stress response, is activated. Indeed, the Met-induced anti-tumor effect was seen to be abrogated in mice with conditional knockout of Nrf2 (47). Glycolysis-dependent activation or elevation of Nrf2, mTORC1 (and/or a proliferation marker Ki67) and p62 occurs in CD8 TILs after Met-treatment, which has been determined by FACS-based monitoring of expression of downstream molecules, such as HO-1, pS6 and p62, respectively.

Importantly, inhibitors of glutaminolysis, and chloroquine, which is an inhibitor of autophagy, blocked the increase in HO-1, pS6 and p62 expression, indicating that glutaminolysis and autophagy are involved. Furthermore, cell-permeable αKG restored chloroquine-dependent depressed HO-1, pS6 and p62, suggesting that selective autophagy-dependent activation of glutaminolysis plays a role in the production of αKG. Glutaminolysis activates mTORC1 via the production of αKG, which promotes the translocation of mTOR to the lysosomal membrane through GTP loading of RagB (48). Activation of mTORC1 results in the phosphorylation of p62 at S351, and p-p62(S351) inhibits Keap1, which allows the translocation of Nrf2 into the nucleus (49). Nrf2 activation triggers selective autophagy that is essentially involved in feeding αKG to the TCA cycle via glutaminolysis and the activation of mTORC1 in CD8 TILs.

Collectively, Met-induced anti-tumor immunity depends on mitochondrial ROS, creating a positive feedback loop, comprising the Nrf2–mTORC1–p62 axis, to ensure the proliferation of CD8 TILs [Fig. 4; Nishida *et al.* (47)]. Because of the lack of nutrients in the TME, Nrf2-dependent activation of selective autophagy does make sense as it supplies glutamine from which αKG is produced via glutaminolysis to fill the TCA cycle and, hence, stimulates anaplerosis. The combination therapy of Met with anti-PD-1 antibody enables robust production of IFNγ from CD8 TILs. It is of note that IFNγ plays a pivotal role in decreasing both glycolysis and OxPhos in tumor cells but not CD8 TILs, as determined by Seahorse analysis. IFNγ receptor (IFNγR) signaling plays a critical role in the down-regulation of the metabolism because the metabolic profile of tumor cells lacking in IFNγR signaling was never affected by IFNγ (47). The underlying mechanism of IFNγ-dependent metabolic down-regulation of tumor cells is unknown.

Inhibition of MCT1 suppresses lactate incorporation in tumor cells *in vivo*. MCTs, including MCT1 and MCT4, are bi-directional transporters, enabling passive transport of lactate in the TME (12, 35, 50). Incorporation of lactate through MCT1 coincides with the import of protons from the same transporter, which reduces the pH within the cell, and promotes the conversion of NAD^+ to NADH (50); hence, generating pyruvate from lactate (Fig. 3). Importantly, the blockade of MCT1 by AZD3965 suppresses the uptake of protons, which elevates intracellular pH. The increase in pH down-regulates the activity of glucose-6-phosphate dehydrogenase (G6PDH), a rate-limiting enzyme for entry into the PPP from glycolysis (51). The PPP is essential for producing NADPH, which contributes to the elevation of the glutathione (GSH)/oxidized glutathione (GSSG) ratio (i.e. reduced/oxidized glutathione) to eliminate

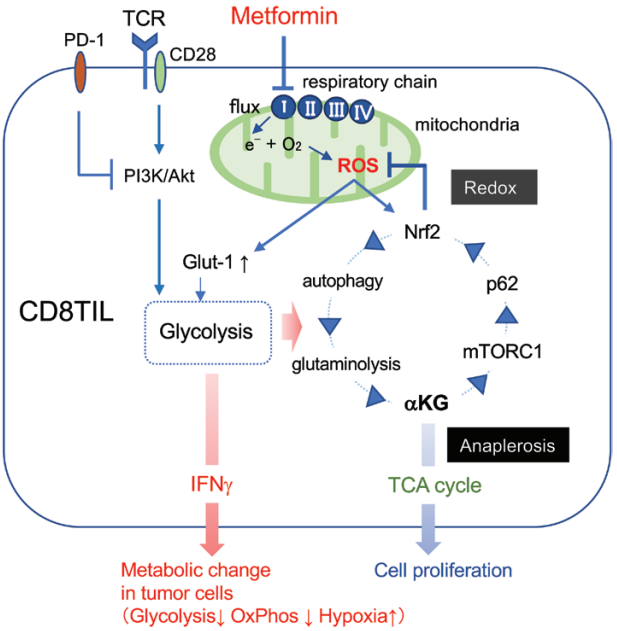

Fig. 4. Met activates glycolysis and anaplerotic reactions involving selective autophagy. Met blocks the transport of electrons (e^-) in the mitochondria, which promotes the regurgitation of the matrix, then the e^- can bind to oxygen to generate O_2^- (superoxide), leading to its conversion to hydrogen peroxide (H_2O_2). The mitochondrial ROS up-regulates Glut-1 expression on the cell surface while activating a positive feedback loop comprising the Nrf2–p62–mTORC1 axis that includes selective autophagy and glutaminolysis. The selective autophagy feeds αKG to the TCA cycle via glutaminolysis (thus, it could be referred to as an anaplerosis pathway), resulting in the activation of mTORC1 in CD8 TILs. Collectively, Met activates glycolysis and the TCA cycle simultaneously, which results in production of IFNγ and proliferation of CD8 TILs, respectively. IFNγ down-regulates glycolysis and OxPhos in tumor cells. Glc, glucose.

ROS. Thus, inhibition of MCT1 by AZD3965 results in the accumulation of oxidative stress.

Tumor cells are very heterogeneous in their metabolism. Highly metastatic tumor cells express more MCT1 on the cell surface to uptake a larger amount of lactate and protons, which in turn activates the TCA cycle (anaplerosis), while decreasing intracellular pH, leading to the activation of the PPP. In other words, the metabolism of highly metastatic tumor cells relies on OxPhos and the cells are more resistant against oxidative stress than less metastatic tumor cells are. Therefore, inhibition of MCT1 by AZD3965 blocks metastasis of melanoma cells, whereas primary tumor growth is not affected (52). Notably, tumor cells in culture depend heavily on glycolysis (10), indicating the production of more lactate that must be exported through MCT1. In this situation, MCT1 inhibition blocks the export of lactate, which results in the suppression of glycolysis and the PPP, leading to cell death (53). The outcome of MCT1 blockade, therefore, depends on the microenvironment surrounding the tumor cells.

Conclusion

It is clear that the polyfunctionality and proliferation of CD8 TILs depend on distinct metabolic pathways—glycolysis and the TCA cycle, respectively. Several anaplerotic pathways,

including FAO, glutaminolysis and glucose-dependent production of pyruvate/acetyl-CoA, support TCA cycle-mediated functions of CD8 TILs to maintain mitochondrial biogenesis. Importantly, pharmacological intervention of these pathways might generate differential consequences for the metabolism of CD8 TILs and tumor cells. Immune cells like CD8 TILs can adapt to pharmacological inhibition and/or activation, but tumor cells might lack such flexibility to change their metabolism, which might substantially contribute to the generation of a favorable TME for immune cells to fight against cancers.

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