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Impaired enolase 1 glycolytic activity restrains effector functions of tumor infiltrating CD8⁺ T cells

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Abstract

In the context of solid tumors, there is a positive correlation between the accumulation of cytotoxic CD8⁺ tumor infiltrating lymphocytes (TILs) and favorable clinical outcomes. However, CD8⁺ TILs often exhibit a state of functional exhaustion limiting their activity, and the underlying molecular basis of this dysfunction is not fully understood. Here, we show that TILs found in human and murine CD8⁺ melanomas are metabolically compromised with deficits in both glycolytic and oxidative metabolism. While several studies have shown that tumors can outcompete T cells for glucose, thus limiting T cell metabolic activity, we report that a down-regulation of the activity of enolase 1, a critical enzyme in the glycolytic pathway, represses glycolytic activity in CD8⁺ TILs. Provision of pyruvate, a downstream product of enolase 1, bypasses this inactivity and promotes both glycolysis and oxidative phosphorylation resulting in improved effector function of CD8⁺ TILs. We found high expression of both enolase 1 mRNA and

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protein in CD8+ TILs, indicating that the enzymatic activity of enolase 1 is regulated post-translationally. These studies provide a critical insight into the biochemical basis of CD8+ TILs dysfunction.

One sentence summary:

Impaired activity of enolase 1 limits glycolysis and effector function of tumor infiltrating CD8+ T cells.

INTRODUCTION

Although the prognostic value of CD8+ tumor infiltrating lymphocytes (CD8+ TILs) in cancer has been reported in various types of cancers(1–3), the progressive loss of proliferative and effector function (exhaustion) of these cells(4, 5) is a major factor in diminishing anti-tumor immunity. The tumor microenvironment (TME) can promote TILs exhaustion via multiple cellular and molecular mechanisms, among which the expression of checkpoint inhibitory molecules, such as PD-L1, have proven clinically tractable. Blocking the inhibitory signals that TILs receive promotes the activation, expansion, and effector activity of TILs(6, 7). Several studies have defined nodes of transcriptional and enzymatic activity that are regulated by checkpoint molecules (8–10), but the underlying biochemical mechanism by which these inhibitors mediate the exhaustion of TILs is still poorly understood. Previous studies showed that the inhibitory checkpoint signals(11) and the TME(12–14) alter metabolic activity of TILs.

There is a strong link between activation-induced proliferation and effector function of T cells and their metabolic activity(15–17). In CD8+ T cells, glucose metabolism is induced initially by TCR signaling upregulating cMYC expression(18, 19) and is sustained by mTORC1-HIF1 α pathway with support from cytokines in a PDK1 dependent manner(20, 21). These signals promote glucose uptake and utilization(22–25). T cell activation induces both glycolytic metabolism and mitochondrial oxidative phosphorylation (OXPHOS), with a more substantial increase occurring in glycolysis(17, 26). Glycolytic metabolism is essential for rapidly dividing cells such as activated T cells, which are thought to trade the ATP production efficiency of OXPHOS for the faster biosynthetic precursor- and ATP-production rate of glycolysis in order to rapidly produce macromolecules and energy(27–29). Notably, T cells that are activated in the absence of glucose(15) or under conditions that prevent them from engaging glycolysis(17) have deficits in their effector function, indicating that glycolytic metabolism contributes to more than the production of essential building blocks. Moreover, T cells with impaired functional activity, such as anergic T cells(30) and exhausted T cells in chronic viral infection(31), are known to have attenuated glycolytic and/or oxidative metabolism. Thus, limited metabolism constrains T cell function.

Recent studies have begun to discern that TILs dysfunction is associated with disrupted glucose metabolism. Competition between tumor cells and CD8+ TILs for the limited amount of glucose in the TME results in attenuated glycolytic metabolism and effector function in CD8+ TILs (11, 13). Further, CD8+ TILs have also been reported to undergo progressive loss of mitochondrial biogenesis and function, in both murine and human

settings (12, 32), limiting ATP production. Notably, enhancing the capacity of *in vitro* activated T cells to produce the glycolytic intermediate, and pyruvate precursor, phosphoenolpyruvate (PEP) increases their anti-tumor activity after adoptive transfer into tumor-bearing mice(13). These studies imply that glucose deprivation prevents T cells from generating the critical glycolytic intermediates that are necessary for T cell function. However, in *ex vivo* studies, dysfunctional TILs retained their low metabolic and functional activities in the presence of supra-physiological level of glucose (11), suggesting the existence of T cell-intrinsic restraint on glycolysis that remains to be elucidated.

To identify the intrinsic regulator in CD8+ TILs glucose metabolism, here we examined the metabolic activity of CD8+ TILs, quiescent CD8+ T cells, and proliferative effector CD8+ T cells (Teff). We found that CD8+ TILs exhibit a post-translational regulation of the critical glycolytic enzyme, ENOLASE 1 (also known as alpha enolase), leading to a deficit in PEP and its downstream metabolite pyruvate. Bypassing ENOLASE 1 by providing of these metabolites partially restored multiple facets of the CD8+ TILs metabolism and effector function. We documented that a combination therapy consisting of CTLA-4, PD-1 and TIM-3 blocking antibodies increased the presence of enolase-active CD8+ TILs in the tumors. We propose that checkpoint blockade can promote the recruitment of CD8+ TILs with higher metabolic activity and thus effector function.

RESULTS

Glucose metabolism by CD8+ TILs is distinct from both quiescent and effector CD8+ T cells.

CD8+ TILs that infiltrate B16cOVA melanoma tumors exhibit phenotypic and functional markers of exhaustion (Fig. S1) in agreement with our previous report of the progressive loss of functional effector CD8+ T cells from the melanoma microenvironment(33). Given the importance of increased cellular metabolism for the proliferation and effector functions of T cells(15–17, 34), we hypothesized that CD8+ TILs are metabolically inactive. To test this, we compared the *ex vivo* metabolic activity of antigen-experienced CD8+ TILs (day 12–15 after tumor implantation) and OVA-specific acute effector CD8+ T cells (Teff, 5 days after vaccination with combination of ovalbumin, α CD40, poly I:C), the latter of which were proliferative and had multiple effector activities. We used naive CD8+ T cells and late (d12) OVA-specific effector CD8+ T cells, whose initial exposure to cognate antigen was synchronized to the time of tumor inoculation, as controls for metabolic quiescence and to control for potential age-related downregulation of metabolic activity. A real-time metabolic assay via extracellular flux (Seahorse) analysis of FACS-sorted CD8+ T cell populations (sorted based on CD44 expression and MHC-dextramer as described in more detail in the Methods) identified that CD8+ TILs had significantly lower ECAR (extracellular acidification rate), a measure of glycolytic metabolism, than acute Teff (Fig. 1A). ECAR measures media acidification as a result of the production of protons from the conversion of pyruvate (the end product of glycolysis) to lactate. These assays were performed in the presence of the physiological concentration of glucose (10 mM) reported in mouse blood and spleen(13) to eliminate glucose availability as a potential limitation on glycolysis. To confirm the attenuated glycolytic metabolism observed in CD8+ TILs, we took advantage of

radiotracer assays in which the consumption of radioactive glucose ($[3\text{-}^3\text{H}]\text{-glucose}$) in glycolysis leads to the liberation of tritiated water ($^3\text{H-H}_2\text{O}$) (35). CD8⁺ TILs produced 5–6-fold less $^3\text{H-H}_2\text{O}$ than acute Teff (Fig. 1B), confirming the low glycolytic activity of CD8⁺ TILs. However, CD8⁺ TILs were glycolytically more active than naive CD8⁺ T cells (Fig. 1B) (which were metabolically quiescent (16)) and late Teff (Fig. 1C).

We next investigated if CD8⁺ TILs have low glycolytic metabolism as a result of preferential reliance on OXPHOS. OXPHOS was determined by quantifying the amount of oxygen consumption from the media (oxygen consumption rate; OCR). We found that CD8⁺ TILs had a lower OCR than acute Teff (Fig. 1D) both under basal conditions and in response to an uncoupler of mitochondrial OXPHOS (FCCP) that allows the determination of maximum OCR. As with ECAR, the OCR of CD8⁺ TILs was higher than that of the naive CD8⁺ T cells and late Teff (Fig. 1E). In agreement with the real-time metabolic analysis, an ATP-dependent luciferase assay showed that CD8⁺ TILs had significantly higher intracellular ATP levels than naive CD8⁺ T cells, but conversely significantly lower levels than acute Teff (Fig. 1F).

CD8⁺ TILs express glucose transporter GLUT1 and efficiently take up glucose.

A potential cause of low metabolic activity in CD8⁺ TILs could be low glucose availability in the tumor microenvironment (11, 13). However, in the *ex vivo* extracellular flux assay system we used pure CD8⁺ TILs and physiological level of glucose (10 times the amount reported in tumor microenvironments), which argued against competition being the sole limiting factor for CD8⁺ TILs metabolism (11, 13). An alternative possibility was that CD8⁺ TILs failed to take up glucose due to compromised expression of transporters. However, qPCR analysis of the mRNA levels of *Glut1* (the major glucose transporter used by T cells (36)) and *Glut3* showed higher expression of these transcripts in CD8⁺ TILs than in naive T cells or Teff (Fig. S2A). Flow cytometric analysis of glucose transporter 1 (GLUT1) protein also identified that the proportion of CD8⁺ TILs that expressed GLUT1 protein was higher than naive CD8⁺ T cells (75%), and similar to Teff (99%, Fig. 2A). On a per cell basis, CD8⁺ TILs expressed the highest amount of GLUT1 (GMFI= 1075) compared to naive CD8⁺ T cells and acute Teff (GMFI= 255 and 820, respectively, Fig. 2A). The expression level of GLUT1 protein on CD8⁺ TILs was also higher than late Teff (Fig. S2B). Consistent with the high level of GLUT1 expression, we found that CD8⁺ TILs were more effective than late Teff and as effective as acute Teff at taking up glucose, as quantified by the uptake of fluorescent glucose analogue 2-NBDG (37) (Fig. 2B). Furthermore, adoptive transfer of *in vitro* activated GLUT1 over-expressing OT-1 (2e6 cells/mouse, activated *in vitro* for 3d with anti-CD3 and anti-CD28) into B16cOVA tumor-bearing mice did not improve tumor control (Fig. 2C). Collectively, these data argued that glucose uptake was not the limiting factor that drove CD8⁺ TILs glycolytic deficiency.

The sustained expression of GLUT1 and glycolytic machinery in CD8 T cells is promoted by contributions of signals from PDK1, mTORC1, and HIF1 α (20, 38, 39) (Fig. 3A). To determine whether CD8⁺ TILs had defects in these signaling components that could affect their glycolytic activity, we performed single cell flow cytometric analysis of phospho-PDK1 (p-PDK1) and phospho-mTORC1 (p-mTORC1) and HIF1 α . Note that PDK1 was

detected using an antibody specific for the autophosphorylation site (S244) and thus reflects PDK1 expression levels rather than any differences in intrinsic activity. We found that these signaling components were activated to a similar level in CD8⁺ TILs and acute Teff. Expression of p-PDK1, p-mTORC1, and HIF1 α were considerably higher in CD8⁺ TILs than late Teff, indicating that CD8⁺ TILs retained the ability to respond to stimuli that can promote the transport and metabolism of glucose (Fig. 3B–D). Given the importance of mTORC1 activity in glycolysis, we further investigated its activity by measuring the levels of p-4E-BP1 and pS6, downstream targets of mTORC1. We found no substantial difference in the activity of mTORC1 between acute Teff and CD8⁺ TILs (Fig. 3E). Taken together, these data suggested that the glycolytic deficiency of CD8⁺ TILs was driven by a cell-intrinsic mechanism that is downstream of glucose uptake and was independent of the canonical signaling pathways that promoted glycolysis.

CD8⁺ TILs possess low levels of phosphoenolpyruvate and enolase activity.

To determine whether a defect lying within the glycolytic pathway and downstream of glucose uptake was responsible for the low metabolic activity of CD8⁺ TILs, we performed a metabolomics analysis (an unbiased assessment of the relative cellular metabolite composition(40)). We identified a significant (~10-fold) reduction in the steady state level of phosphoenolpyruvate (PEP) in CD8⁺ TILs compared to acute Teff (Fig. 4A). The relative abundance of the glycolytic metabolites upstream of PEP was similar (Fig. 4A and Fig. S3A), implying that the PEP production step of glycolysis is the point at which glycolysis is disrupted in CD8⁺ TILs. Furthermore, low levels of pyruvate were found in CD8⁺ TILs, arguing that excessive PEP to pyruvate conversion did not account for the low levels of PEP (Fig. 4A). Therefore, we hypothesized that PEP deficiency in CD8⁺ TILs was mediated by reduced expression or activity of ENOLASE 1, the enzyme that is responsible for PEP production in glycolysis. Surprisingly, qPCR and western blot analysis of enolase 1 in FACS-purified CD8⁺ T cell populations demonstrated that the mRNA and protein levels of *eno1*/ENOLASE 1 were similar between acute Teff and CD8⁺ TILs (Fig 4B). Intracellular flow cytometric analysis confirmed that equivalent proportions of Teff and TILs express ENOLASE 1, and that the level of ENOLASE 1 expression in acute Teff and CD8⁺ TILs were similar (Fig 4C). We also found that the mRNA levels of many glycolytic enzymes were equivalent between acute Teff and CD8⁺ TILs (Fig. S3B and S3D). However, significantly higher amounts of the mRNAs of glycolytic enzymes were observed in CD8⁺ TILs than in age-matched d14 Teff (Fig. S3C and S3D), confirming the notion that CD8⁺ TILs were not metabolically quiescent as shown previously in Fig. 1C and E. Together, these data demonstrated that the glycolytic metabolism of CD8⁺ TILs was altered at the level of PEP production by factors other than the availability of the precursor metabolites or the enzymes responsible for production of PEP.

To understand whether the ENOLASE 1 protein in CD8⁺ TILs had compromised enzymatic activity that could result in PEP deficiency, we directly assessed enolase activity in lysates prepared from FACS-sorted CD8⁺ T cell populations. We found a striking deficit in enolase activity in CD8⁺ TILs compared to acute Teff (Fig. 4E, left). Furthermore, enolase activity did not increase in FACS-sorted CD8⁺ TILs following a strong *in vitro* T cell stimulation achieved by cross-linking TCR with anti-CD3 in the presence of anti-CD28. In contrast, *in*

vitro T cell stimulation resulted in a marked upregulation of the enolase activity in FACS-sorted acute Teff assessed in parallel (Fig. 4E, right). Taken together, these data supported the hypothesis that CD8+ TILs glycolytic metabolism was restrained by enolase activity rather than enolase expression, implying that enolase function was regulated at the post-translational level in CD8+ TILs.

Overcoming PEP deficiency rescues metabolic and functional activity of CD8+ TILs

During glycolysis, PEP serves as a substrate for synthesis of pyruvate, which can promote both glycolysis via generation of NAD⁺ in a metabolic program known as aerobic glycolysis (the Warburg effect) and also fuels OXPHOS through the mitochondrial tricarboxylic acid (TCA) cycle(28). Given the low glycolytic and OXPHOS metabolism, despite high glucose uptake, that we observed in CD8+ TILs, we speculated that providing exogenous pyruvate (bypassing glycolysis, or more specifically overcoming the PEP shortage) should rescue CD8+ TILs aerobic glycolysis and OXPHOS. Indeed, exogenous pyruvate provided during metabolic flux analysis of FACS-sorted, ex vivo CD8+ TILs significantly increased the CD8+ TILs basal ECAR, yet had no impact on acute Teff basal ECAR (Fig. 5A–B, Basal ECAR). The lack of effect on exogenous pyruvate on acute Teff presumably occurred because these cells produced sufficient pyruvate. Notably, provision of pyruvate also substantially increased the maximal ECAR achieved by CD8+ TILs (measurement of ECAR in the presence of oligomycin, which inhibits OXPHOS and forces cells to be dependent upon glycolysis for ATP production, Fig. 5A–B, Max. ECAR). Interestingly, exogenous pyruvate also enhanced both the basal and maximal OXPHOS in CD8+ TILs as well as acute CD8+ Teff (Fig. 5A–B, Basal OCR and Max. OCR), suggesting that glycolytic or pyruvate deficiency could be limiting the OXPHOS capability of CD8+ TILs. Importantly, during a 4hr *in vitro* stimulation, the addition of pyruvate resulted in a significant increase in the frequency of CD8+ TILs that produced IFN γ and TNF α (Fig. 5C). Similar results were observed when we provided exogenous PEP to the CD8+ TILs during the short term *in vitro* cultures (Fig. 5D), suggesting that PEP shortage impairs CD8+ TILs function by limiting pyruvate availability. Together, these data indicated that low enolase activity impaired the metabolic and contributed to the lowered functional activity observed in CD8+ TILs.

To further interrogate the contribution of enolase activity to CD8+ T cell function, we investigated cytokine production after treating Teff and CD8+ TILs with sodium fluoride (NaF), a well-studied enolase inhibitor(41, 42). Consistent with the reported inhibitor effect of NaF on enolase activity in other cells types, NaF strongly inhibited enolase activity in Teff when added to cell culture during acute *in vitro* stimulation (Fig. S4A, cells). Furthermore, NaF completely inhibited enolase activity (Fig. S4A, lysate) when added to the lysates of untreated Teff 5 min prior to assay, suggesting that the reduction in enolase activity as a result of the NaF treatment of cell culture was due to the direct effect of NaF on enolase. Notably, NaF substantially reduced cytokine production from Teff after *in vitro* culture with peptide-pulsed antigen presenting cells (Fig. S4B–C), further supporting the importance of enolase activity to the effector function of T cells. Importantly, NaF also significantly reduced the remaining cytokine production from CD8+ TILs (Fig. S4B–C and Fig 5C), indicating that although it was relatively weak, the activity of enolase in CD8+ TILs played a significant role in supporting the residual effector function observed in these

cells. Taken together, these results indicated that deficiency in enolase activity limited the effector function of CD8⁺ TILs.

Immune checkpoint blockade therapy recruits enolase-active CD8⁺ TILs into tumors

PD1 has been reported to inhibit glycolytic metabolism in human CD4⁺ T cells(43). Consistent with this, a recent study has shown that blockade of either PD-1 or CTLA-4 augments the glycolytic metabolism in CD8⁺ TILs responding to mouse sarcoma tumors(11). This led us to ask if immune checkpoint signals constrained enolase activity in CD8⁺ TILs. We treated mice bearing tumors established for nine days with a combination of checkpoint blocking antibodies (anti-PD1, anti-CTLA4 and anti-TIM3 every three days). This treatment regimen increased the absolute number of CD8⁺ TILs in the tumors (Fig. 6A) and constrained tumor outgrowth (Fig. 6B) compared to controls. This combinatorial checkpoint molecule inhibition (CPi) did not have significant effect on the expression of enolase 1 (Fig. 6C–D). However, the CPi treatment significantly improved enolase activity in CD8⁺ TILs (Fig. 6E). To determine the relative contribution of the inhibition of PD1, CTLA-4, or TIM3 to the increased enolase activity, we treated tumor-bearing mice with antibodies targeting individual checkpoint molecules. Checkpoint monotherapies did not significantly improve enolase activity in CD8⁺ TILs (Fig. 6F). Together, these results indicated that multiple checkpoint signals contributed to the low enolase activity observed in CD8⁺ TILs.

To understand the contribution of enolase activity to the anti-tumor effects of the CPi-treated CD8⁺ TILs, we investigated the impact of inhibiting enolase on the cytokines and lytic molecules (perforin) production by the CPi-treated CD8⁺ TILs. Treatment of CPi-treated CD8⁺ TILs with NaF significantly reduced the capacity of the CPi-treated CD8⁺ TILs to produce IFN γ and perforin (Fig. S5A–B). These data suggested that the increase in enolase activity in the CPi-treated CD8⁺ TILs contributed to the anti-tumor activity of these cells.

We next investigated whether the CPi treatment increased enolase activity in CD8⁺ TILs via reactivation of enolase in pre-existing intratumoral CD8⁺ TILs or by inducing/sustaining enolase activity in newly infiltrating CD8 TILs. For this study, the CPi experiment was conducted in the presence or absence of a well-known S1PR-agonist FTY-720 that blocks T cells from exiting the secondary lymphoid tissues. FTY-720 treatment abrogated the ability of CPi treatment to increase enolase activity in CD8⁺ TILs (Fig. 6G). These data indicated that the combinatorial checkpoint molecule blockade either enhanced enolase activity in the recently activated CD8⁺ TILs or sustained it in the newly tumor infiltrating T cells, rather than re-activating enolase in the CD8⁺ TILs that are already in the tumors.

CD8⁺ TILs isolated from human melanomas have low enolase activity

To understand whether enolase activity is altered in CD8⁺ TILs that infiltrate the human cancers, we sorted antigen experienced (CD45RO⁺) CD8⁺ TILs from metastatic melanoma samples. Compared to PBMC from healthy donors that were stimulated *in vitro* to generate Teff, human CD8⁺ TILs produced limited amount of IFN γ after cross-linking the TCR (Fig. S6A). Activation of human healthy donor PMBC also strongly induced (4-fold) enolase activity as judged by direct analysis of enolase within cell lysates (Fig. S6B). Over 80% of

the antigen-experienced human CD8⁺ TILs (Fig. 6H) and about 50% of the antigen-experienced CD4⁺ TILs expressed ENOLASE 1 (Fig. S6D)). However, consistent with our murine studies, we found limited enolase activity in both antigen-experienced CD8⁺ TILs and CD4⁺ TILs compared to the acutely activated human healthy donor T cells (Fig. 6I and Fig. S6E). Notably, there was a trend towards higher enolase activity in 41BB (CD137)-positive compared to 41BB-negative CD8⁺ TILs (Fig. S6C), where 41BB expression identified T cells that may have recently responded to TCR stimulation(44). This suggested that functional enolase may correlate with T cells that maintain functional responsiveness. Thus, we conclude that human melanoma CD8⁺ TILs have similar low enolase activity as observed in murine models, and that their metabolic activity is likely to be similarly impeded, contributing to the observed defect in effector function.

DISCUSSION

Elevation of metabolic activity is essential for effector T cells to support their proliferation and function(19, 22, 31). Our data support the developing notion that an underlying basis of TILs dysfunction is insufficient glycolysis and OXPHOS to support strong effector functions(11–14). This state persists even though CD8⁺ TILs are capable of efficiently transporting glucose and have active signaling pathways that normally promote the expression of the enzymes that are involved in glucose metabolism. This indicates that CD8⁺ TILs represent a population of cells that are undergoing constant stimulation and that their attenuated metabolic activity is not a function of immunological quiescence. Rather, glucose metabolism is constrained in CD8⁺ TILs by weak enolase activity, which resulted in limited production of PEP, a vital glycolytic intermediate, and provision of pyruvate or PEP is sufficient to restore some of the metabolic and functional activity of CD8⁺ TILs. Remarkably, the reduction in enolase activity is regulated post-translationally. Our data show that a checkpoint molecule inhibition protocol that slowed tumor outgrowth results in the infiltration of tumor by CD8⁺ TILs with improved enolase activity. The low proliferative and functional activity of CD8⁺ TILs, allied with their limited metabolic activity, could indicate that these cells are simply quiescent. However, compared to naive and late (age-matched) effectors, we find high levels of expression and activation of the PDK1-mTORC1-HIF1 α signaling pathway, which lies downstream of the TCR and cytokine receptors. Indeed, the level of activation of this pathway was equal to, or higher, than seen in acute Teff. Consistent with this, we found no defect in the expression of GLUT1, the major transporter of glucose in lymphocytes, or the ability of CD8⁺ TILs to acquire glucose from their surroundings. Thus, the inability of CD8⁺ TILs to consume glucose despite the expression of the glycolytic machinery indicates that the glycolytic inactivity of CD8⁺ TILs is regulated by a functional defect in the glycolytic pathway rather than quiescence or a lack of stimulation. Notably, while the biomarkers (i.e. signaling molecules and molecular components of glycolysis) of T cell glycolysis correlate well with the actual metabolic activity (as judged by flux assays) of naive and effector CD8⁺ T cells, it is important to note that there is not a strong correlation between the activation state of these molecules and the overall metabolic activity of CD8⁺ TILs; thus, the sole use of glycolytic biomarkers may over-estimate the metabolic activity of CD8⁺ TILs. Importantly, this study pinpointed the mechanism responsible for restraining CD8⁺ TILs glycolytic metabolism to weak enolase

activity, which resulted in reduced levels of PEP and pyruvate. PEP can support T cell function in two major ways: by promoting cytokine production via calcium signaling(13) and by serving a substrate for pyruvate synthesis(28). A recent study has demonstrated that the anti-tumor activity of *in vitro* activated T cells after adoptive transfer into tumor-bearing mice improves when PEP production is augmented(13). Our findings not only support that report, but also directly show that endogenous CD8+ TILs have low levels of PEP and that the shortage of this metabolite constrains both the glycolytic and mitochondrial metabolism in CD8+ TILs. Surprisingly, we did not observe accumulation of glycolytic metabolites upstream of PEP as one might expect from the loss of enolase activity. However, this is not uncommon in a complicated biological systems such as glycolysis, which involves bidirectional reactions, feedback inhibition, and metabolites with multiple possible fates(27, 28). For example, 2-PG (enolase's substrate) can be converted back to upstream metabolite 3-PG(47), which can be taken out of glycolysis and be used for nucleotide and lipid synthesis via serine biosynthesis(28, 48).

Our studies have found no deficiency in enolase protein expression, indicating that post-translational regulation likely impedes enolase enzymatic activity. There are several potential mechanisms of regulation of enolase activity in CD8+ TILs. First, post-translational modifications of ENOLASE 1 have been described, including phosphorylation, acetylation, and methylation(49), though it is unclear whether these modifications influence enolase enzymatic activity. Second, enolase enzymatic activity requires divalent cations and is inhibited by fluoride (50), and thus may be susceptible to ion availability in the tumor microenvironment. Third, in addition to enolase 1's typical cytoplasmic localization where it performs its enzymatic role, ENOLASE 1 can also be directed to the cell surface where it serves as a receptor for plasminogen(49); is recruited to mitochondria where it preserves mitochondrial integrity and membrane potential(51); or expressed as an alternative translational product (Myc Binding Protein -1) that is translocated to the nucleus(56). Thus, the inactivity of enolase in CD8+ TILs may either be a result of regulation of its enzymatic activity or repurposing of ENOLASE 1 away from glycolysis. The precise regulatory mechanism that lead to enolase activity of TILs remains to be determined. We also do not understand whether the low enolase activity in CD8+ TILs is a function of weak priming or loss within the tumor. We found that concomitant blocking of multiple immune checkpoint molecules increased the presence of CD8+ TILs with more active enolase and led to a decrease in tumor outgrowth. The increase in enolase-active CD8+ TILs that occurs with CPI was determined to arise from newly infiltrating T cells. We interpret these data to mean that CPI either promotes enolase activity during CD8+ T cell priming or preserves enolase activity in the newly infiltrating CD8+ T cells as they enter the tumor. Currently, there is no assay with sufficient sensitivity to assess the enolase or glycolytic activity of CD8+ TILs in the early stages of their response to tumors in the draining lymph node. Sensitive metabolic assays are also required to investigate whether the weak enolase activity observed in the murine and human TILs is only apparent in those cells embedded within the tumor, or whether tumor-specific or bystander T cells within the stroma, at the periphery of the tumor, or within the circulation are also metabolically compromised.

Our current study identifies augmentation of enolase activity as an avenue for increasing TILs function, though it should be noted that the therapies targeting the combination of

checkpoint molecules used in this study have yet to be evaluated clinically. We have concluded that the limited glycolysis and enolase activity exhibited by CD8⁺ TILs contributes to the remaining effector function of these cells as NaF eliminated remaining activity. Although NaF is a well-characterized inhibitor of enolase, we acknowledge that it could have other, enolase-independent impacts on T cells. Further, blocking checkpoint signals or isolating CD8⁺ TILs from the tumors (as done in the *in vitro* studies) did not fully restore enolase activity, suggesting the existence of additional as yet unidentified pathways regulating enolase and glycolysis. While PEP and pyruvate were found to enhance TILs function *in vitro* here and in our previous studies on renal cell carcinoma(32), bypassing enolase inactivity in vivo with PEP or pyruvate may be challenging as PEP does not cross the cell membrane and pyruvate may be used by tumors. The most likely immediate clinical utility of pyruvate rescue approach will be to support the metabolic activity of TILs or PBMC that are intended to be used in adoptive cell transfer protocols. These limitations of PEP- and pyruvate-based rescue approaches highlight the need to understand how enolase activity is impaired in CD8⁺ TILs.

Recent studies by others(12) and the data presented here show a low level of OXPHOS in CD8⁺ TILs. While this could be attributed to low mitochondrial mass or mitochondrial membrane potential(12), the ability of pyruvate to rescue the OXPHOS in CD8⁺ TILs raises intriguing questions about the contribution of pyruvate deficiency to the loss of mitochondrial function. Interesting, ENOLASE 1 has been previously reported to bind the mitochondrial membrane and prevent loss of membrane potential, which is essential for the OXPHOS(51). Whether mitochondrial binding of ENOLASE 1 supports mitochondrial function by bringing together pyruvate synthesis and mitochondria is an intriguing question that will need to be addressed. In conclusion, restoration of ENOLASE 1 activity might be expected not only to promote glycolysis but also improve the OXPHOS capabilities of TILs, together substantially improving effector activity and durability within tumors.

MATERIALS AND METHODS

Study design

The objectives of the study were to determine whether the dysfunction of murine and human melanoma CD8⁺ TILs is a function of cell-intrinsic defects in glycolytic metabolism that results in curtailed effector functions and to identify the mechanism that may attenuate the glycolytic metabolism, which could be targeted or used as a biomarker for translation purpose. For murine studies, C57BL/6 mice were either injected with B16cOVA melanoma cells or immunized with OVA protein, poly IC, and anti-CD40. Cohort sizes were determined by power studies defined using prior tumor outgrowth kinetics. Tumors were allowed to develop for 2–3 weeks or until they reached the maximum size (for tumor growth studies) permitted by the University of Virginia's Animal Care and Use Committee protocol. Tumor size was measured in an un-blinded manner. CD8⁺ TILs were isolated from the tumors while naive CD8⁺ T cells and antigen-specific effector CD8⁺ T cells were harvested from the spleens of the immunized, non-tumor bearing mice. All the metabolic studies were done on ex vivo, FACS-sorted T cells to remove competition for nutrients with tumor cells and also the impact of tumor and other cells on the CD8⁺ T cells. For checkpoint blockade

studies, tumor-bearing mice received three doses of control IgG or a combination of anti-PD-1, anti-CTLA-4, and anti-TIM3 every three days starting on day 8 post-tumor implantation. Tumors were harvested one day after the last treatment for analysis. In the studies involving human samples, antigen experienced CD8⁺ and CD4⁺ T cells were isolated from the tumors of melanoma patients and PBMC of healthy subjects under informed consent in compliance with UVA Human Subjects IRB # 10598. All animal studies were done in accordance with the University of Virginia Animal Care and Use Committee protocol # 3292

Tumor cell line, mice, and tumor injection

Ovalbumin-expressing B16 melanoma cell line (B16cOVA) was previously generated in our laboratory(54) and was maintained in RMP11640 (ThermoFisher) supplemented with 5% FBS (Gemini Bio) in the presence of selection agent (blastocidin, Invitrogen). 4×10^5 B16cOVA in 100 μ l HBSS were injected subcutaneously into shoulders or flank of male C57BL/6 mice purchased from National Cancer Institute. Tumors were allowed to develop for 2–3 weeks before harvesting for analysis. All mice were treated in accordance to procedures established by the University of Virginia Animal Care and Use Committee protocol # 3292.

Human Samples

Metastatic melanoma samples were collected with informed consent under protocol UVA-IRB # 10598. Excised tumors were processed by the UVA Biorepository and Tissue Procurement Facility (BTRF) Core using a protocol of an initial non-enzymatic disaggregation followed by sequential incubations in a mixture of DNase/collagenase/hyaluronidase (55)). Non-digested and digested fractions were stored in the liquid nitrogen. Anonymous male and female healthy donor PBMC were generated from Buffy Coats purchased from Research Blood Components. PBMC were isolated from the Buffy coat by density gradient centrifugation using Ficoll–Hypaque plus (VWR). PBMC were cultured with 10 IU/ml IL-2 alone or in combination with anti-CD3 (1 μ g/ml plate bound) and anti-CD28 (1 μ g/ml in solution), as indicated in the Figure Legends. The activity of enolase in the lysates of the human Teff and TILs was assessed after FACS sorting the *in vitro* stimulated healthy donor PBMC and the ex vivo human melanoma TILs (non-digested tumor fractions) for live, CD8⁺ or CD4⁺ T cells that are CD3⁺CD45RO⁺ and CD137⁺ or CD137⁻. All sorts were performed under BSL-2 conditions using Becton Dickinson Influx cell sorter by the UVA Flow Cytometry Core.

Checkpoint blockade treatment

Tumor-bearing mice received no treatment, i.p. injection of 300 μ g of control IgG (Equitech-Bio, Inc.) or a combination of 100 μ g i.p. of each of anti-PD1 (RMP1–14), anti-TIM3 (RMP3–23) (both from Dr Hideo Yagita, Juntendo University), and anti-CTLA4 (9H10, BioXcell) with or without 2 μ g/ml FTY-720 on days 8, 11, and 14 post-tumor injection. The FTY-720 treatment was continuously provided through the drinking water starting on day 8 post-tumor injection. Tumor-bearing mice were treated with 250 μ g of each of antibodies in the monotherapy studies.

Adoptive T cell transfer

OT-I T cells were *in vitro* stimulated with plate-bound anti-CD3 and soluble anti-CD28 (both at 5 µg/ml) and transferred (2 million cells/mouse i.v.) into day 5 B16cOVA tumor-bearing mice. Tumor size was measured every three days with caliper. Mice were euthanized when tumor size reached 2000mm³.

T cell preparation, staining, and flow cytometric analysis

Splenocytes were isolated from the spleens of naive mice or mice that were immunized with combination of 500 µg ovalbumin (Sigma), 75 µg poly I:C (Invitrogen), and 100 µg anti-CD40 (clone FGK45, provided by Dr. Stephen Schoenberger, La Jolla Institute) by mechanical homogenization followed by either density gradient separation with Lympholyte-M (Cedarlane) or RBC lysis (Affymetrix). For mouse TILs isolation, intact tumors were excised from mice and subjected to mechanical homogenization and density gradient separation as above. Samples were then stained with Live/Dead Aqua (Invitrogen), OVA₂₅₇-dextramer (Immudex) and fluorescent conjugate antibody specific to surface proteins (CD8, CD44). Following surface staining, samples were fixed/permeabilized with 4% paraformaldehyde/0.1% Tween 20 in 1× PBS or cytofix/cytoperm (BD) then stained with antibodies specific to intracellular proteins: phosphorylated 3-Phosphoinositide-dependent protein kinase 1 (p-PDK1, Ser244, Santa Cruz), p-mTORC1 (Ser2448, cell signaling), p-S6 (Ser235/236, Cell signaling), p-4E-BP1 (Thr37/46, cell signaling), HIF1α (IC1935P, R&D system), PKM2 (D78A4, cell signaling), GLUT1 (EPR3915, Abcam), ENOLASE 1 (EPR10863(B), Abcam), IFNγ (XMG1.2, ThermoFisher), TNFα (MP6-XT22, Biolegend) and secondary goat anti-rabbit FITC (ab6009, Abcam). FOXP3 fixation/permeabilization buffer and nuclear staining protocol (ThermoFisher) was used to stain for HIF1α (241812, R&D systems) and Ki67 (SolA15, ThermoFisher).

T cells were *in vitro* stimulated with 1 or 5 µg/ml anti-CD3 (specified in figure legends) or 10 µg/ml OVA₂₅₇ peptide-pulsed, CD45-mismatched splenocytes for 4hr prior to cytokine staining or for 1hr prior to pS6 and p4E-BP1 staining. For PEP or pyruvate functional rescue experiments, cells were treated with DMSO (vehicle), 1 µg/ml PEP (Sigma), or 1mM pyruvate (ThermoFisher) for 10 min on ice in glucose-free XF minimal media (Agilent). 0.005% saponin (Sigma) was added to partially permeabilized cells to facilitate PEP entry, as previously described(13). Cells were then washed with 1× PBS and incubated at 37°C in CO₂-free incubator in the XF minimal media for 10 min to let the cells rest prior to proceeding to 4hr stimulation and cytokine staining. In the experiment involving analysis of cytokine production after acute inhibition of enolase activity, cells were *in vitro* stimulated with 1 µg/ml anti-CD3 and 10 IU/ml IL-2 for 24hrs in the presence of vehicle control (water) or 2mM sodium fluoride (NaF). Brefeldin A (BFA) was added only for the last 4hrs of the cell culture to enhance cytokine staining.

Flow cytometric analysis of the stained cells was conducted by using FACS Canto II (BD Scientific) or Cytoflex (Beckman Coulter) cytometers. Data were analyzed using FlowJo software (Version 10, TreeStar). To identify the T cells population of interest, we gated on live cells, lymphocytes, singlets, and CD8+ cells followed by CD44-low and dextramer-negative for naive T cells; CD44-high and dextramer-positive for Teff and TILs unless

differently stated in the figure legend, as in the case of some *in vitro* restimulation experiments where total CD44hi Teff and TILs were taken due to the impact of TCR stimulation on dextramer staining. For T cell sorting, single cell suspensions of spleens or tumors were initially enriched for CD8+ T cells by MACS magnetic bead separation (Miltenyl Bioscience), then stained with OVA₂₅₇-dextramer and antibody against surface antigens as above and placed in 4,6-diamidino-2-phenylindole (DAPI, Sigma) containing media sorted by using Influx cell sorter (Becton Dickenson) applying stringent gating criterion to achieve >99% purity.

Glucose uptake

Splenocytes and TILs samples were prepared as described above and subjected to live/dead staining in 1× PBS at 4 °C for 20 min followed by OVA₂₅₇-dextramer staining in 5% FBS at 4 °C for 30min. Cells were then incubated at 37°C and 5% CO₂ for 1hr in glucose-free DMEM media (ThermoFisher) containing antibodies specific to surface proteins for cell identification. Cells were then washed with the glucose-free DMEM media, further incubated under the above culture condition for 15min in DMEM media with or without 100 μM 2-NBDG (ThermoFisher), and then washed in 1× PBS and immediately analyzed by flow cytometry while keeping samples on ice (37).

Analysis of enolase activity in *in vitro* and *ex vivo* T cell samples

Cell lysates were prepared according to the enolase activity kit protocol (Sigma) from FACS-sorted CD8+ T cells and CD8+ TILs directly *ex vivo*. Alternatively, T cells were cultured *in vitro* for 4 days with either IL-2 alone (10 IU/ml) or with 5 μg/ml plate-bound anti-CD3, 2 μg/ml soluble anti-CD28, and 10 IU/ml IL-2. The lysates were then stored at -80 °C until fluorometric direct enolase activity (PEP formation) assay was conducted according to the manufacturer's instructions. Fluorescent intensity was read at $\lambda_{ex} = 535/\lambda_{em} = 587$ nm for 45–60min at 3min interval by using Spectramax M5 plate reader (Molecular Devices) set to kinetic reading. In the experiments involving the inhibition of enolase activity, CD8+ T cells from the spleens of the immunized mice were restimulated with 1 μg/ml plate-bound anti-CD3, 2 μg/ml soluble anti-CD28, and 10 IU/ml IL-2 in the presence of 2 μM NaF or vehicle control (water). Alternatively, lysates prepared from *in vitro* CD8+ Teff generated as above were treated with 2 μM NaF or vehicle control for 5 min prior to reading enolase activity.

Metabolic assay

Naïve and effector CD8+ T cells and CD8+ TILs were FACS sorted as described above, washed with 1×PBS, and seeded (2×10^5 cells/well) in XFp cell culture miniplate in XF minimal media (Agilent) supplemented with 10 mM glucose (Sigma), 2 mM glutamine (Invitrogen), with or without 2 mM sodium pyruvate (ThermoFisher). ECAR and OCR were measured by extracellular flux analysis using XFp (Agilent) in the response to 1 μM oligomycin, 1 μM fluoro-carbonyl cyanide phenylhydrazone (FCCP), and 0.5 μM rotenone/antimycin (all from Sigma except antimycin, MP Biochemical). Radioactive-based measurement of glycolytic flux was performed through quantification of the amount of ³H-H₂O released from [3-³H]-glucose during glycolysis as described previously(35). Intracellular ATP levels of FACS-sorted naïve CD8+ T cells, Teff, and CD8+ TILs was

determined by using luciferase-based ATP Bioluminescence Assay Kit HS II (Cat# 11699709001, Roche). Cell lysates were prepared and processed for ATP measurement according to the assay kit protocol. The ATP-dependent light emitted during the luciferase reaction was measured by luminometer (Zylux Femtomaster FB15, Zylux).

Metabolomics

FACS-sorted naïve, effector and tumor-infiltrating CD8⁺ T cells were washed in RPMI plus 10% FBS, resuspended in pre-chilled 80% methanol/water on dry ice and transferred to -80 °C freezer for 15 min to disrupted cells membrane and quench enzymatic activities. Samples were then vortexed while being kept cold and spun down at 16,000 rcf in table top centrifuge to extract the metabolites. Supernatants were harvested and dried by SpeedVac. Samples were then processed and subjected to LC-MS analysis as previously described (40)

RNA extraction, cDNA synthesis, and real-time qPCR

Total RNA was extracted from FACS-sorted naïve CD8⁺ T cells, effector CD8⁺ T cells, and CD8⁺ TILs with RNeasy plus Kit (QIAGEN), and then reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio- Rad). SYBR Green-based real-time qPCR analysis was performed using CFX96 Detection System (Bio-Rad). All samples were run in biological triplicates and normalized to ribosomal protein S18 (Rps18). Fold change in gene expression level was determined by ddCt method comparing the expression level of each sample to that of naïve CD8⁺ T cells. The sequences of the primers are as follows:

Gene	Forward primers	Reverse primers
RPS18S	5'-ATGCGGC GGC GTTATTCC-3'	5'-GCTATCAATCGTTCAATCCTGTCC-3'
GLUT1	5'-CAGTTCGGCTATAACACTGGTG-3,	5'-GCCCCGACAGAGAAGATG-3'
mGlut3	TAAACCAGCTGGGCATCGTTGTTG	AATGATGGTTAAGCCAAGGAGCCC
Hk2	5'-TGATCGCCTGCTTATTCACGG-3'	5'-AACCGCCTAGAAATCTCCAGA-3'
Pfkfb	5'-GGAAAGGAAGACAGAGTGGGAGGC-3'	5'-CAGATCGACCTCAACAGTGGGATTC-3'
Pfkfb	5'-GGTACAGATTCAGCCCTGCACC-3'	5'-GTCGGCACCAGCAAGTCAAGG-3'
GAPDH	GTCGGTGTGAACGGATTG	5'-TAGACTCCACGACATACTCAGCA-3'
Pgm3	5'-CCCAGCATCTCGATCATATCATGTTTCG-3'	5'-GTCCTGCTCCTCCGCACTGG-3'
Eno1	5'-GGAAAGGAAGACAGAGTGGGAGGC-3'	5'-CAGATCGACCTCAACAGTGGGATTC-3'
Ldha	5'-CATTGTCAAGTACAGTCCACACT-3'	5'-TTCCAATTA-CTCGGTTTTTGGGA-3'
Ldha	5'-GGACAAGTGGGTATGGCATGTG-3'	5'-CCGTCAACCACCAATCTTAGA-3'
MCT4	5'-TCACGGGTTTCTCCTACGC-3'	5'-GCCAAAGC-GGTTACACAC-3'

Statistical Analysis

All data are presented as mean $\pm$ SEM. Statistically significances were determined by unpaired Student's t-test when comparing two groups and by one-way ANOVA or two-way ANOVA, followed by Tukey's Multiple Comparison Test when comparing more than two groups. GraphPad Prism 7 was used to perform the statistics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. CD8⁺ TILs are metabolically less active than acute effector CD8⁺ T cells, but more active than quiescent CD8⁺ T cells.

(A-F) Naive and antigen-specific effector (day 5 and day 12) CD8⁺ T cells were FACS sorted from the spleens of C57Bl/6 mice immunized with OVA protein, poly I:C, and anti-CD40, while antigen-experienced CD8⁺ TILs were FACS sorted from B16cOVA melanoma tumors that developed subcutaneously in C57Bl/6 mice for 12–15 days. (A-C) *Ex vivo* T cell glycolytic metabolism as measured by the extracellular acidification rate (ECAR) (A and C) or the amount tritiated water (³H₂O) released from [3-³H]-glucose during glycolysis (B). The basal ECAR was measured in unmanipulated cells while the maximum ECAR was

quantified after exposing cells to a mitochondrial ATP synthase inhibitor (1 μ M oligomycin) during the extracellular flux assay. (D-E) Oxidative metabolism (OXPHOS) of the cells in A and C as quantified by oxygen consumption rate (OCR). Basal OCR from sorted cells in the steady state while maximum OCR are cells in response to exposure to an uncoupler of mitochondrial OXPHOS (1 μ M FCCP). (F) Intracellular ATP levels in sorted ex vivo naive CD8⁺ T cells, day 5 (d5) Teff, and d14 CD8⁺ TILs as measured by luciferase-based ATP determination kit. Data in A, C, D, and E are representative of at least three independent experiments where samples were sorted from n = 3–5 mice per group then pooled in each experiment. Data used to generate graph in B were compiled from two independent experiments where the indicated sorted T cell samples were pooled from n = 5 – 9 mice per group. Data in F are representative of three independent experiments with samples pooled from n=3–5 mice per group in each experiment. Data in A and D show mean $\pm$ SEM, and analyzed by unpaired Student's *t*-test. Data in B, C, E and F show mean $\pm$ SEM, and analyzed by one-way ANOVA, followed by Tukey's Multiple Comparison Test. **p* <0.05, ***p* <0.01, ****p* <0.001.

Fig. 2. Glucose uptake is not limited in CD8⁺ TILs.

(A) Flow cytometric analysis of glucose transporter 1 (GLUT1) protein expression by *ex vivo* naive CD8⁺ T cell, d5 Teff, and d14 CD8⁺ TILs generated as in Figure 1. The numbers within representative dot plots describe the frequency of cells expressing GLUT1 for individual samples. Bar charts show the overall frequency (middle chart) and intensity of GLUT1 expression (GMFI, right chart), of GLUT1⁺ cells in CD8⁺CD44^{lo} naive CD8⁺ T cells and OVA-specific CD8⁺CD44^{hi} for d5 Teff and CD8⁺ TILs. (B) Glucose uptake potential of *ex vivo* d5 and d14 Teff and d14 CD8⁺ TILs as measured by flow cytometry after pulsing cells with the fluorescent glucose analog, 2-NBDG. The frequency (middle chart) of 2-NBDG⁺ cells and amount of uptake (right chart) within the OVA-specific CD8⁺CD44^{hi} cell population are shown. (C) Survival of B16cOVA tumor bearing mice was assessed after adoptive transfer of *in vitro* activated non-transgenic or GLUT1 transgenic OT-I T cells five days after s.c. tumor injection. Data in A and B are representative of three to five independent experiments, with at least two mice per group in each experiment. Data in C are from n=7 mice per group, and the experiment was repeated twice. Data show mean $\pm$ SEM: one-way ANOVA, followed by Tukey's Multiple Comparison Test. *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 3. Glycolysis-regulating enzymes are expressed and active in CD8⁺ TILs.

(A) The signaling pathway downstream of TCR and cytokine signals that are known to promote the glucose uptake and glycolysis. (B-D) Flow cytometric analysis of phosphorylated PDK1 (p-PDK1, Ser244), p-mTORC1 (Ser2448), and HIF1α expression in naive CD8⁺ T cells and OVA-specific d5 Teff, d14 Teff, and d14 CD8⁺ TILs that were prepared as in Fig. 1. (C) GMFI of pPDK1, p-mTORC1, and HIF1α in the CD8⁺CD44^{lo} naive or OVA-specific CD8⁺CD44^{hi} Teff and TILs expressing the protein of interest. (D) The frequency of p-PDK1⁺, p-mTORC1⁺, or HIF1α⁺ cells within naive CD8⁺CD44^{lo} and

OVA-specific CD8⁺CD44^{hi} Teff and TILs. (E) Phosphoflow cytometric analysis of mTORC1 activity via determination of the phosphorylated downstream targets (S6 and 4E-BP1) after 1 hr *in vitro* stimulation of acute Teff and CD8⁺ TILs with 5 µg/ml anti-CD3. The CD8⁺ TILs *in vitro* stimulation was also conducted in the presence of 250 µM rapamycin to inhibit pS6 or 250 µM Torin-1 to inhibit p4E-BP1 for staining negative control. Data in B-D are representative of three to five independent experiments, with at least two mice per group in each experiment. Data in E are from samples pooled from n = 5–6 mice per group, and the experiment was repeated twice. Data show mean ± SEM: one-way ANOVA, followed by Tukey's Multiple Comparison Test. *p <0.05, **p<0.01, ***p<0.001.

Fig. 4. Weak enolase activity restrains glucose metabolism in glycolysis in CD8+ TILs.

(A) Metabolomic analysis of the relative metabolite composition of d5 Teff and d14 CD8+ TILs normalized to that of naive CD8+ T cells. Metabolites were extracted from similar samples to that described in Figure 1 and subjected to mass spectrometric analysis. PEP, phosphoenolpyruvate; 3-PG/2-PG, 3-phosphoglycerate and 2-phosphoglycerate. (B) The expression of *enolase 1* transcripts in d5 Teff and d14 CD8+ TILs was measured by qPCR and protein by western blot (numbers indicate molecular weight). (C-D) Flow cytometric analysis of ENOLASE 1 protein expression in naive CD8 T cells, d5 Teff, and d14 CD8+ TILs. The frequencies of cells that express ENOLASE 1 within naive CD8+CD44^{lo} and

OVA-specific CD8+CD44^{hi} Teff or CD8+ TILs are indicated. GMFI is derived from ENOLASE 1-expressing cells. (E) Fluorescence-based spectrophotometric analysis of enolase activity (PEP formation) measured in lysates prepared from FACS-sorted naive CD8+ T cells, acute Teff, and CD8+ TILs either directly ex vivo (left) or after *in vitro* stimulation with anti-CD3 and anti-CD28 for 4 days (right). Data are on samples pooled from n = 5–9 mice per group (A), representative from two independent experiments with samples pooled from at least six mice per group (B), and are representative of three independent experiments with at least three mice per group (C-D). Data in E is combined from multiple independent experiments with T cells pooled from 2–3 mice per group. Data show mean ± SEM, and analyzed by unpaired student's *t*-test (A, B and E, right) or one-way ANOVA, followed by Tukey's Multiple Comparison Test (D and E, left). **p* < 0.05, ***p* < 0.01, ****p* < 0.001

Fig. 5. Bypassing enolase restores metabolic function and effector activity in CD8+ TILs. (A and B) ECAR and OCR by FACS-sorted d5 Teff and d12 CD8+ TILs as measured by Seahorse in pyruvate-free or 2mM pyruvate-containing XF minimal media. Basal ECAR and basal OCR were measured without any manipulation while maximum ECAR and maximum OCR were determined after oligomycin or FCCP exposure, respectively. (C-E) Intracellular flow cytometric analysis of IFN γ and TNF α production from Teff and CD8+ TILs treated for 4hrs with vehicle, 5mM pyruvate, or 2 μ M NaF during in vitro culture and stimulation with OVA257-pulsed antigen presenting cells. (D) Fold increase in the proportion of CD8+

T cell expressing cytokines after culturing with 5mM pyruvate or 1 µg/ml PEP (after partial permeabilization) for 4hrs. Data are representative of two to three independent experiments and show mean $\pm$ SEM. Statistical analyses were done by unpaired student's t-test (A, B and D), and two-way ANOVA, followed by Tukey's Multiple Comparison Test C. *p <0.05, **p<0.01, ***p<0.001.

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Fig. 6. Checkpoint molecule blockade supports CD8⁺ TILs enolase function.

(A - G) B6 mice were s.c. injected with B16cOVA tumor cells and treated with either control, combination of checkpoint inhibitors (CPI: anti-PD1, anti-CTLA4, and anti-TIM3) antibodies or individual antibodies on days 8, 11, and 14. (G) FTY-720 treatment was continuously provided through the drinking water starting on day 8 post-tumor injection. All tumors were harvested on day 15 post-injection. (A) The absolute numbers of antigen experienced CD8⁺ TILs per mm² tumor. (B) Tumor growth over time. (D) The frequency (left and middle) and intensity of expression (right) of enolase within OVA-specific CD8⁺CD44^{hi} populations from control or combined CPI-treated mice. (E-G) Fluorescent-based spectrophotometric direct enolase activity (PEP formation) measurement in the lysates prepared from FACS-sorted antigen-experienced CD8⁺ TILs from control, combined-CPI (E

and G), or individual-CPi (F) treated mice in the absence (E-G) or presence of FTY720 (G). The enolase activity in panel E and F were normalized to the average of the control replicates for CD8+ TILs to allow cross-experiment comparison. (H and I) Analysis of ENOLASE 1 expression and activity in ex vivo human CD8+ TILs using human healthy donor PBMC that were *in vitro* stimulated with anti-CD3 and anti-CD28 for three days as a positive control. (H) Frequency of ENOLASE 1 positive cells within CD8+CD45RO+ human Teff and TILs. (I) Fluorescent-based spectrophotometric analysis of the activity of enolase in lysates prepared from FACS-sorted CD8+CD45RO+ human Teff and TILs. Data are representative of three independent experiments (A-D), combined from three to five independent experiments with samples pooled from at least three mice per group (E and F), combined from two independent experiments with at least three mice per group (G), or from two healthy donor PBMC and three melanoma patient TILs (H-I). Data show mean $\pm$ SEM and analyzed by unpaired student's t-test. *p <0.05, **p<0.01, ***p<0.001.