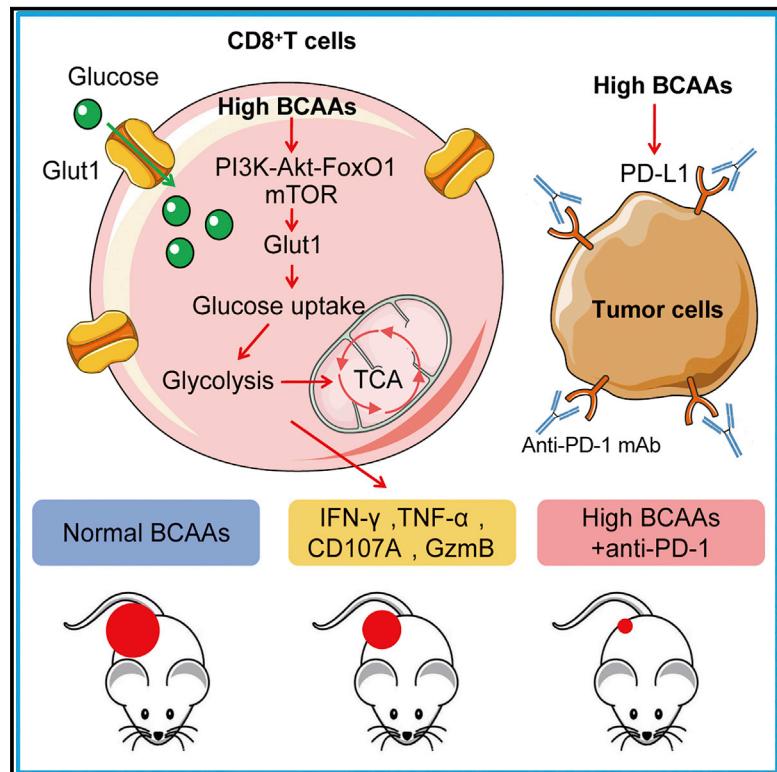


Accumulation of branched-chain amino acids reprograms glucose metabolism in CD8⁺ T cells with enhanced effector function and anti-tumor response

Graphical abstract



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In brief

Yao et al. report that BCAA accumulation in CD8⁺ T cells upregulates Glut1 expression and increases glucose uptake, leading to reprogramming CD8⁺ T cell metabolic features prone to anti-tumor immunity. BCAs might become alternative supplementary components to increase the clinical efficacy of immune checkpoint inhibitors.

Highlights

- CD8⁺ T cells from *PP2Cm*^{-/-} mice exhibit high BCAA content and enhanced functionality
- BCAA accumulation in CD8⁺ T cells promotes Glut1 expression and glucose uptake
- BCAA supplementation magnifies CD8⁺ T cell effector with altered glucose metabolism
- BCAs synergize with anti-PD-1 regimen in anti-tumor immunity



Article

Accumulation of branched-chain amino acids reprograms glucose metabolism in CD8⁺ T cells with enhanced effector function and anti-tumor response

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SUMMARY

Branched-chain amino acids (BCAAs) provide nutrient signals for cell survival and growth. How BCAAs affect CD8⁺ T cell functions remains unexplored. Herein, we report that accumulation of BCAAs in CD8⁺ T cells due to the impairment of BCAA degradation in 2C-type serine/threonine protein phosphatase (PP2Cm)-deficient mice leads to hyper-activity of CD8⁺ T cells and enhanced anti-tumor immunity. CD8⁺ T cells from PP2Cm^{-/-} mice upregulate glucose transporter Glut1 expression in a FoxO1-dependent manner with more glucose uptake, as well as increased glycolysis and oxidative phosphorylation. Moreover, BCAA supplementation recapitulates CD8⁺ T cell hyper-functions and synergizes with anti-PD-1, in line with a better prognosis in NSCLC patients containing high BCAAs when receiving anti-PD-1 therapy. Our finding thus reveals that accumulation of BCAAs promotes effector function and anti-tumor immunity of CD8⁺ T cells through reprogramming glucose metabolism, making BCAAs alternative supplementary components to increase the clinical efficacy of anti-PD-1 immunotherapy against tumors.

INTRODUCTION

CD8⁺ T cells are key components to exert immunity against infection and cancers through perforin/granzyme (Gzm)-mediated cytotoxicity and cytokine production including interferon (IFN)- γ and tumor necrosis factor (TNF)- α , etc. During the activation, proliferation, and differentiation processes, metabolic reprogramming in CD8⁺ T cells is one of the key mechanisms to determine the destiny of immune responses.¹ When naive T cells are activated, they undergo a metabolic shift from oxidative phosphorylation (OXPHOS) to glycolysis for energy support and biomass production, while fatty acid oxidation is the main metabolic property for the maintenance of memory CD8⁺ T cells.² Accordingly, intrinsic transcriptional,³ post-transla-

tional,⁴ epigenetic regulations,⁵ as well as extrinsic nutrition factors,⁶ are demonstrated to be extensively dedicated to metabolic modulation of CD8⁺ T cell differentiation and function. In the tumor microenvironment (TME), metabolic fitness is also critical for anti-tumor immunity of local immune cells including CD8⁺ T cells. To overcome the impairment of tumor-infiltrating T cells either due to the upregulation of co-inhibitors or by suppressive cytokines and surrounding cells etc.,⁷ seeking metabolic strategies to preserve the functionality, especially in nutrient-limited TME is of great necessity for CD8⁺ T cell-mediated anti-tumor immunity.

Branched-chain amino acids (BCAAs), including leucine, isoleucine, and valine, are essential amino acids obtained only from external diet supplementation.⁸ They are building blocks



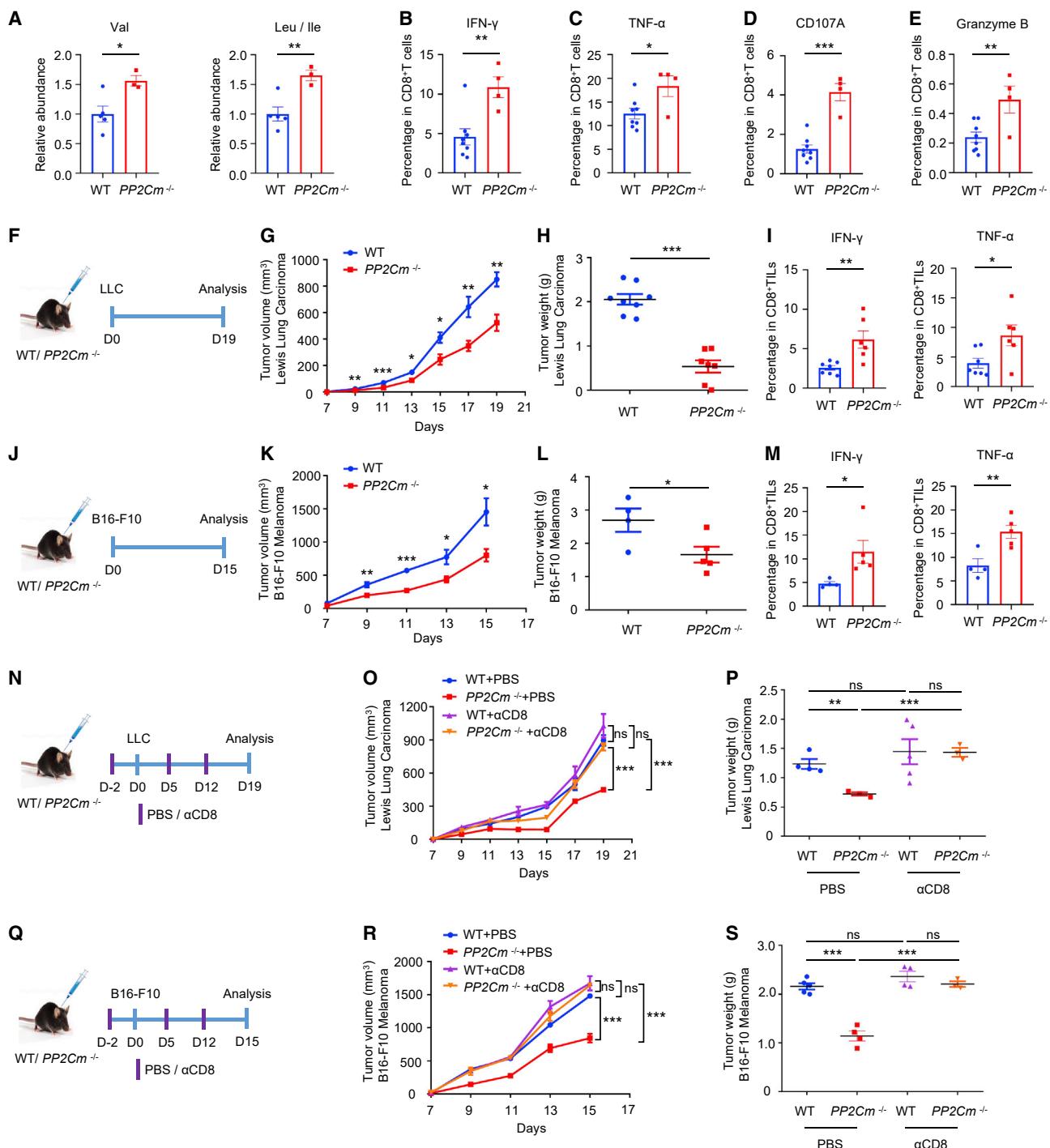


Figure 1. PP2Cm^{-/-} mice exhibit enhanced CD8⁺ T cell functionality and anti-tumor immunity

- (A) Comparisons of intracellular Val (left) and Leu/Ile (right) levels in CD8⁺ T cell from WT (n = 5) versus PP2Cm^{-/-} (n = 3) mice.
- (B–E) Percentages of IFN- γ ⁺ (B), TNF- α ⁺ (C), CD107A⁺ (D), and GzmB⁺ (E) CD8⁺ T cells in the spleens of WT (n = 8) versus PP2Cm^{-/-} (n = 4) mice.
- (F) Experimental scheme.
- (G and H) LLC tumor growth curves (G) and tumor weight at day 19 (H) in WT (n = 8) versus PP2Cm^{-/-} mice (n = 7). Data from three independent experiments were pooled and presented.
- (I) Percentages of IFN- γ ⁺ and TNF- α ⁺ CD8⁺ T cells in LLC tumors from WT (n = 7) versus PP2Cm^{-/-} mice (n = 6). Data from two independent experiments were pooled and presented.
- (J) Experimental scheme.

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for *de novo* synthesis and structural maintenance of endogenous proteins to maintain cell survival and cell growth.⁹ BCAAs also serve as the precursors for biosynthesis of sterol and keto bodies.¹⁰ They can further provide nutrient signals to regulate cellular metabolism and cell growth in a mammalian target of Rapamycin (mTOR)-dependent manner.¹¹ Being carbon and nitrogen sources, BCAA catabolism is initiated by the transamination of BCAAs into branched-chain α -keto acids (BCKAs) that are catalyzed by cytosolic or mitochondrial branched-chain aminotransferases (BCATs). BCKAs are then oxidized by branched-chain α -keto acid dehydrogenase (BCKD) complex and eventually degraded into the derivatives of coenzyme A (CoA), such as acetyl-CoA and succinyl-CoA.¹² In the BCAA catabolic pathway, BCKD is the bottleneck enzyme.¹³ Its activity can be regulated by a mitochondrial 2C-type serine/threonine protein phosphatase (PP2Cm)¹⁴ through directly binding to the BCKD complex for the activation.¹⁵ Therefore, *PP2Cm* deficiency leads to elevated BCAAs in the periphery of *PP2Cm*-deficient mice.¹⁶

It has been widely reported that abnormal BCAA catabolism was associated with many diseases, including metabolic disorders,^{17,18} heart diseases,¹⁹ chronic liver diseases,²⁰ and certain malignancies.²¹ Investigations from heart failure models indicate that *PP2Cm* deficiency induces an increase in intracellular BCAA and BCKA levels, leading to mTOR activation, reactive oxygen species (ROS) production, and more susceptibility to calcium-induced permeability transition pore opening in cardiac myocytes.²² Abnormal BCAA degradation is dedicated to cell proliferation in certain neoplasms such as glioma,²³ acute myeloid leukemia,²⁴ pancreatic duct adenocarcinoma (PDAC),²⁵ and hepatocellular carcinomas.²⁶ BCAAs are also capable of regulating immune responses. Accumulation of intracellular Leu activates the mTOR complex 1 (mTORC1) signaling pathway, which leads to the hyperactivation of CD4⁺ T cells.²⁷ Our previous study showed that *BCAT1* was upregulated and *PP2Cm* was downregulated in human CD8⁺ T cells following T cell receptor (TCR) triggered activation.²⁸ However, how the availability of BCAAs affects the differentiation and function of CD8⁺ T cells with metabolic adaptation has not been addressed. A better understanding of how CD8⁺ T cells responding to BCAA availability may aid in direct modulation of immune response against infection and cancers.

In this study, we reported that CD8⁺ T cells from either *PP2Cm*^{-/-} mice or BCAA-supplemented mice with accumulation of BCAAs exhibited hyper-activity with enhanced cytokine production and cytotoxicity as well as anti-tumor immunity. The underlying mechanisms of CD8⁺ T cell hyper-activity were investigated together with synergistic roles of BCAAs in PD-1

blockade therapy, which might shed light on the potential of BCAAs as an alternative nutrient support in combination immunotherapy.

RESULTS

PP2Cm^{-/-} mice exhibit enhanced CD8⁺ T cell functionality and anti-tumor activity

Our previous studies reported that the expressions of key enzymes related to BCAA catabolism including *BCAT1* and *PP2Cm* have been altered upon TCR/CD28 stimulation in human CD8⁺ T cells (Figure S1), implying the associations between BCAA catabolism and CD8⁺ T cell activation. In *PP2Cm*^{-/-} mice we have constructed previously,¹³ there exhibited increased levels of intracellular (Figure 1A) and plasma (Figure S2A) BCAAs. No obvious defects in T cell development in the thymus were observed in *PP2Cm*^{-/-} mice (Figure S2B). However, there were slightly increased CD8⁺ T cell proportions in the spleens when compared with wild-type (WT) littermates (Figure S2C). What is more, the proportions of IFN- γ , TNF- α , and granzyme B (GzmB) secreting CD8⁺ T cells as well as CD107A⁺CD8⁺ T cells were exaggerated significantly in *PP2Cm*^{-/-} mice (Figures 1B–1E), illustrating the enhancement of CD8⁺ T cell effector functionality in *PP2Cm*^{-/-} mice at steady states. Alternatively, the percentages of CD4⁺ T cells, B cells, and natural killer (NK) cells (Figures S2D–S2F), the production of IFN- γ and TNF- α in CD4⁺ T cells (Figures 2G and 2H) and GzmB⁺ NK cells (Figure S2I) were mostly comparable between WT and *PP2Cm*^{-/-} mice. We further examined anti-tumor responses in *PP2Cm*^{-/-} mice by using syngeneic Lewis lung carcinoma (LLC)-grafted tumor models (Figure 1F). There was no difference in body weight between WT and *PP2Cm*^{-/-} mice before and after being challenged with LLC cells (Figure S2J). *PP2Cm*^{-/-} mice displayed remarkable retardation in tumor growth with less tumor weight when compared with WT mice (Figures 1G and 1H). Tumor-infiltrating CD8⁺ T cells from *PP2Cm*^{-/-} mice displayed increased IFN- γ and TNF- α production (Figure 1I), which was similar in the spleens from LLC-transplanted *PP2Cm*^{-/-} mice (Figure S2K and S2L), as well as GzmB production (Figure S2M) when compared with those from WT littermates. Similarly, in B16-F10-grafted mice, tumor growth was slowed down (Figures 1J and 1K) and tumor weight was reduced (Figure 1L) while IFN- γ and TNF- α production in CD8⁺ T cells were increased (Figure 1M) in *PP2Cm*^{-/-} mice as compared with those in WT counterparts. To determine whether CD8⁺ T cells are involved in tumor suppression in *PP2Cm*^{-/-} mice, an anti-mouse CD8 monoclonal antibody (mAb) was used (Figure 1N) to deplete CD8⁺ T cells in *PP2Cm*^{-/-} and WT mice

(K and L) B16-F10 tumor growth curves (K) and tumor weight at day 15 (L) in WT ($n = 4$) versus *PP2Cm*^{-/-} ($n = 5$) mice.

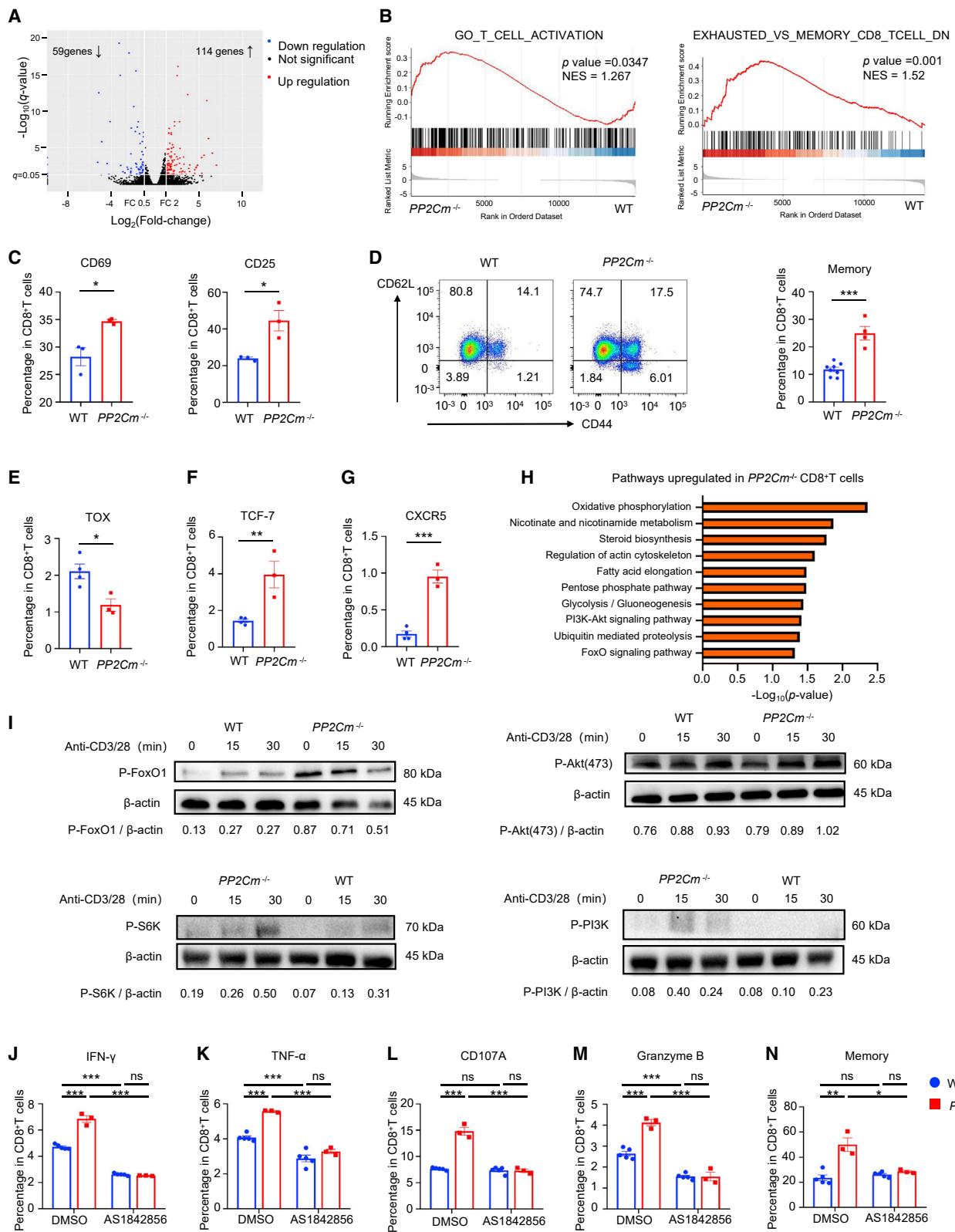
(M) Percentages of IFN- γ ⁺ and TNF- α ⁺ CD8⁺ T cells in B16-F10 tumors from WT ($n = 4$) versus *PP2Cm*^{-/-} mice ($n = 5$).

(N) Experimental scheme.

(O and P) LLC tumor growth curves (O) and tumor weight (P) in WT + PBS ($n = 4$), *PP2Cm*^{-/-} + PBS ($n = 3$), WT + anti-CD8 ($n = 5$) or *PP2Cm*^{-/-} + anti-CD8 ($n = 3$) mice.

(Q) Experimental scheme.

(R and S) B16-F10 tumor growth curves (R) and tumor weight (S) in WT + PBS ($n = 5$), *PP2Cm*^{-/-} + PBS ($n = 4$), WT + anti-CD8 ($n = 4$) or *PP2Cm*^{-/-} + anti-CD8 ($n = 3$) mice. Data are presented as mean \pm SEM. ns, non-significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by the Student's t test (A–S). Unless indicated, data are representative of two independent experiments.



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(Figure S2N). We found that the depletion of CD8⁺ T cells abrogated the suppression of tumor growth in *PP2Cm*^{-/-} mice with similar tumor growth and tumor weight to those in WT mice (Figures 1O and 1P). Similarly, tumor growth of B16-F10 cells in CD8⁺ T cell-depleted *PP2Cm*^{-/-} mice (Figure S2O) was also accelerated to similar levels in WT mice (Figures 1Q–1S). These results demonstrate that with the accumulation of BCAAs in *PP2Cm*^{-/-} mice, CD8⁺ T cells exhibit enhanced effector function and anti-tumor immunity.

CD8⁺ T cells from *PP2Cm*^{-/-} mice possess activation and memory-like features with the activation of mTOR and PI3K-AKT-FoxO1 signaling axis

To further elucidate molecular signatures related to the enhancement of CD8⁺ T cell functionality in *PP2Cm*^{-/-} mice, we performed RNA sequencing (RNA-seq) transcriptome analysis on purified WT and *PP2Cm*^{-/-} CD8⁺ T cells upon anti-CD3/CD28 stimulation. There were 114 genes upregulated and 59 genes downregulated significantly in *PP2Cm*^{-/-} CD8⁺ T cells when compared with those from WT mice (Figure 2A). Gene set enrichment analysis (GSEA) plots showed the enrichment of activation (Figure 2B, left, false discovery rate [FDR]-adjusted p = 0.0347) and memory-like signatures (Figure 2B, right, FDR-adjusted p = 0.001). Consistently, higher proportions of CD69⁺ and CD25⁺CD8⁺ T cells were present in the spleens from *PP2Cm*^{-/-} mice when compared with WT littermates (Figure 2C), as well as more CD44⁺CD8⁺ memory T cells (Figure 2D). The expression of exhaustion-related transcription factor like TOX (Figure 2E) was reduced, whereas TCF-7 (Figure 2F) and CXCR5 (Figure 2G), the indicators for T cell stemness,²⁹ were upregulated in CD8⁺ T cells from *PP2Cm*^{-/-} mice. Similarly, tumor-infiltrating CD8⁺ T cells from LLC-implanted *PP2Cm*^{-/-} mice displayed more TCF-7 expression, and reduced PD-1, Tim-3, and TOX expressions as well (Figures S3A–S3D).

In addition, KEGG pathway enrichment analysis revealed that signaling pathways associated with cell survival and energetic metabolisms were significantly upregulated in *PP2Cm*^{-/-} CD8⁺ T cells after *in vitro* activation when compared with WT counterparts (Figure 2H). Since the mTOR pathway is widely reported to be regulated by BCAAs,³⁰ accumulation of BCAAs in *PP2Cm*^{-/-} CD8⁺ T cells also activated mTOR signaling demonstrated by increased phosphorylation of ribosomal protein S6, an indicator of mTORC1 activation, upon anti-CD3/CD28 stimulation (Figure 2I). PI3K-AKT-FoxO1 signaling pathway has been demonstrated previously to integrate metabolic and mitogenic signals in CD4⁺ T cells,³¹ which was also upregulated in *PP2Cm*^{-/-} CD8⁺ T cells based on RNA-seq data. We have observed that

PP2Cm^{-/-} CD8⁺ T cells had elevated phosphorylation of PI3K, AKT^{S473}, and FoxO1 when compared with WT counterparts (Figure 2I) as well. Moreover, the addition of AS1842856, a FoxO1 inhibitor,³² led to a dramatic decrease in IFN-γ and TNF-α production (Figures 2J and 2K), as well as the reduction of CD107A and GzmB (Figures 2L and 2M) in *PP2Cm*^{-/-} CD8⁺ T cells to similar levels of WT CD8⁺ T cells. The percentages of CD44⁺CD8⁺ memory T cells also decreased to similar levels in WT counterparts (Figure 2N). Although the addition of Rapamycin, an mTORC1 inhibitor, led to a dramatic decrease in IFN-γ and TNF-α as well as CD107A and GzmB in *PP2Cm*^{-/-} CD8⁺ T cells, they were still higher when compared with WT CD8⁺ T cells (Figures S3E–S3H). These data indicate that CD8⁺ T cells from *PP2Cm*^{-/-} mice exhibit activation and memory-like features with hyper-activity of the PI3K-AKT-FoxO1 signaling axis, which is largely dedicated to enhanced immunoactivity.

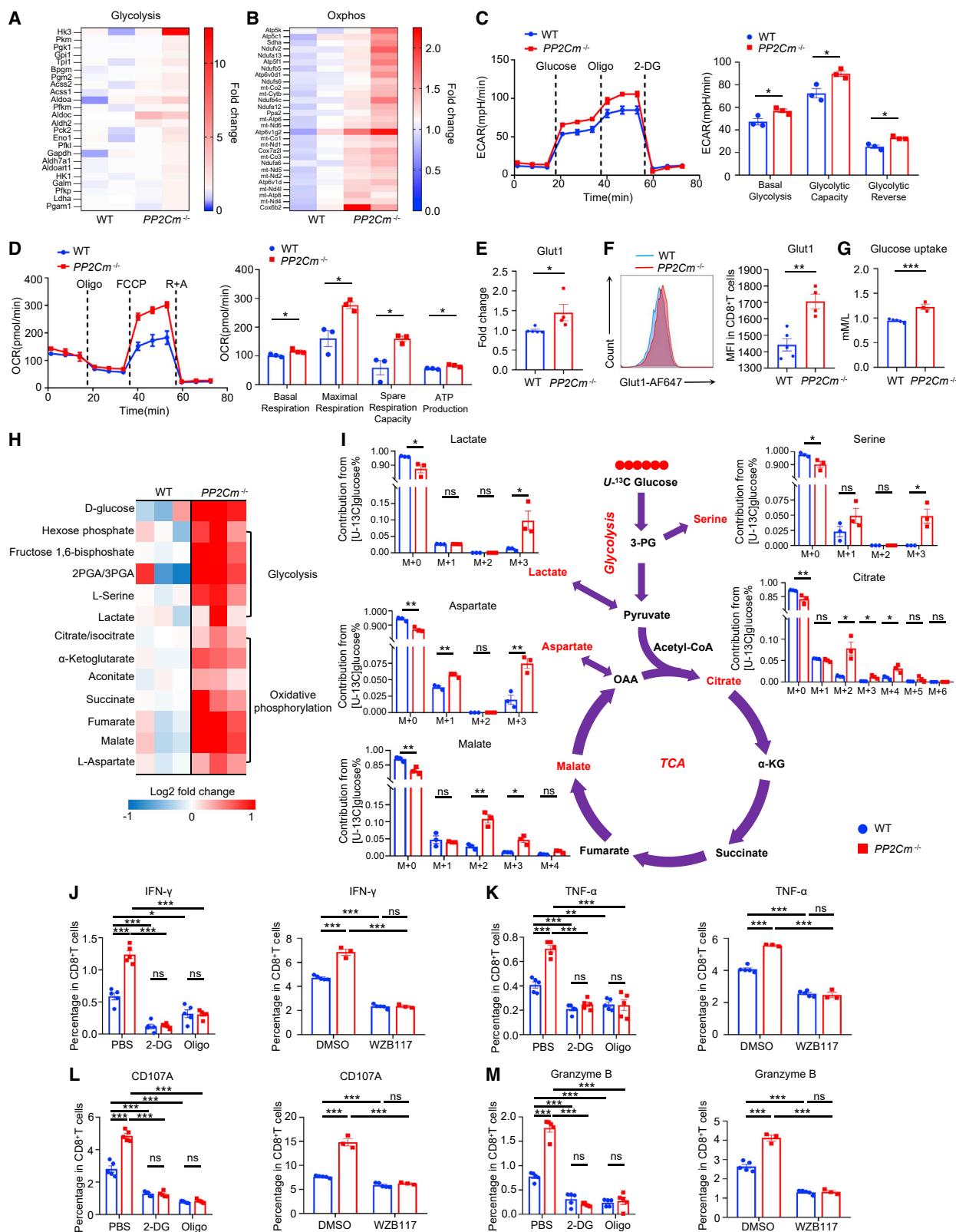
PP2Cm^{-/-} CD8⁺ T cells display highly activated glycolysis and OXPHOS

The transcriptomic analysis also showed that genes involved in glycolysis and OXPHOS were upregulated in *PP2Cm*^{-/-} CD8⁺ T cells (Figures 3A and 3B) with the enrichment of relevant gene signature plots from GSEA analysis (Figures S3I and S3J). Consistently, increased extracellular acidification rate (ECAR) occurred in *PP2Cm*^{-/-} CD8⁺ T cells with significantly elevated values of basal glycolysis, glycolytic capacity, and glycolytic reverse when compared with WT CD8⁺ T cells (Figure 3C). *PP2Cm*^{-/-} CD8⁺ T cells also showed significantly higher mitochondrial oxygen consumption rate (OCR) with more mitochondrial spare respiratory capacity than WT CD8⁺ T cells (Figure 3D). What is more, the expression level of glucose transporter 1 (Glut1), which is a key glucose importer on CD8⁺ T cells,³³ was significantly increased in *PP2Cm*^{-/-} CD8⁺ T cells as compared with WT CD8⁺ T cells both at the mRNA levels and protein levels demonstrated by RT-PCR and a small shift in flow cytometric analysis respectively (Figures 3E and 3F). Meanwhile, *PP2Cm*^{-/-} CD8⁺ T cells consumed more glucose than WT CD8⁺ T cells upon anti-CD3/CD28 stimulation (Figure 3G), indicating an increase in glycolytic flux inside *PP2Cm*^{-/-} CD8⁺ T cells.

Therefore, we further performed a comparative metabolomic analysis on activated CD8⁺ T cells from *PP2Cm*^{-/-} versus WT mice using a liquid chromatograph-mass spectrometer (LC-MS) (Figure S4A). Not surprisingly, BCAA accumulation was detectable in *PP2Cm*^{-/-} CD8⁺ T cells. Furthermore, we found that the concentrations of intracellular intermediates

Figure 2. *PP2Cm*^{-/-} CD8⁺ T cells possess gene signatures with activation and memory-like features and hyperactivation of mTOR-PI3K-AKT-FoxO1 signaling axis

- (A) Volcano plots indicating the alterations of gene expressing profiles in WT versus *PP2Cm*^{-/-} CD8⁺ T cells.
- (B) GSEA analysis indicating the activation (left) and overlapping exhausted versus memory CD8⁺ T cell down sites (right).
- (C) Percentages of CD69⁺ and CD25⁺ CD8⁺ T cells in the spleens of WT (n = 3) versus *PP2Cm*^{-/-} (n = 3) mice.
- (D) Representative flow plots (left) and quantifications (right) of the percentages of memory CD8⁺ T cells in WT (n = 8) versus *PP2Cm*^{-/-} mice (n = 4).
- (E–G) Percentages of TOX⁺ (E), TCF-7⁺ (F), CXCR5⁺ (G) CD8⁺ T cells in the spleens of WT (n = 4) versus *PP2Cm*^{-/-} (n = 3) mice.
- (H) Enrichment of KEGG pathways upregulated in CD8⁺ T cells from *PP2Cm*^{-/-} mice compared with WT counterparts.
- (I) Immunoblotting of P-S6K, P-PI3K, P-AKT (473), and P-FoxO1 in WT and *PP2Cm*^{-/-}CD8⁺ T cells upon anti-CD3/CD28 stimulation. The expression levels of target proteins were normalized with corresponding β-actin.
- (J–N) Percentages of IFN-γ⁺ (K), TNF-α⁺ (L), CD107A⁺(M), GzmB⁺ (N), and CD44⁺ (O) CD8⁺ T cells upon treatment with AS1842856 versus DMSO. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 by the Student's t test (C–G, J–N). Data are representative of two independent experiments.



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involved in glycolytic pathway including hexose-6-phosphates, fructose-1,6-bisphosphate (FBP), and 3-phosphoglycerate (3PGA) were increased in *PP2Cm*^{-/-} CD8⁺ T cells (Figure 3H). An accumulation of pyruvate was also detectable together with an elevated extracellular level of lactate in *PP2Cm*^{-/-} CD8⁺ T cells. In addition, we observed a significant increase in tricarboxylic acid (TCA) cycle intermediates including citrate, α -ketoglutarate (α -KG), succinate, fumarate, and malate in *PP2Cm*^{-/-} CD8⁺ T cells (Figure 3H). Amino acids like serine and aspartate that could be synthesized from glycolytic and TCA cycle intermediates were also accumulated in *PP2Cm*^{-/-} CD8⁺ T cells (Figure S4A). Moreover, the tracing experiments with uniformly labeled [*U*-¹³C] glucose (Figure 3I) showed a notable increase in the fractions of glycolysis-derived serine and lactate, and TCA cycle-related citrate, aspartate, and malate in *PP2Cm*^{-/-} CD8⁺ T cells when compared with WT counterparts. When we added WZB117, a Glut1 inhibitor,³⁴ to CD8⁺ T cells during anti-CD3/CD28 stimulation, the activities of glycolysis and OXPHOS were substantially suppressed in *PP2Cm*^{-/-} CD8⁺ T cells with dramatic decreases in relevant key intermediates (Figures S4B–S4J). When WZB117, 2-deoxyglucose (2-DG), and oligomycin (Oligo), which are the inhibitors of Glut1, glycolysis, and OXPHOS, respectively, were added during the activation of CD8⁺ T cells, there resulted in an apparent reduction of *PP2Cm*^{-/-} CD8⁺ T cells expressing IFN- γ , TNF- α , CD107A, and GzmB to the base levels of WT CD8⁺ T cells (Figures 3J–3M). Therefore, upregulation of glycolysis and OXPHOS initiated from Glut1 over-expression and more glucose uptake is dedicated to enhanced effector function of CD8⁺ T cells in *PP2Cm*^{-/-} mice.

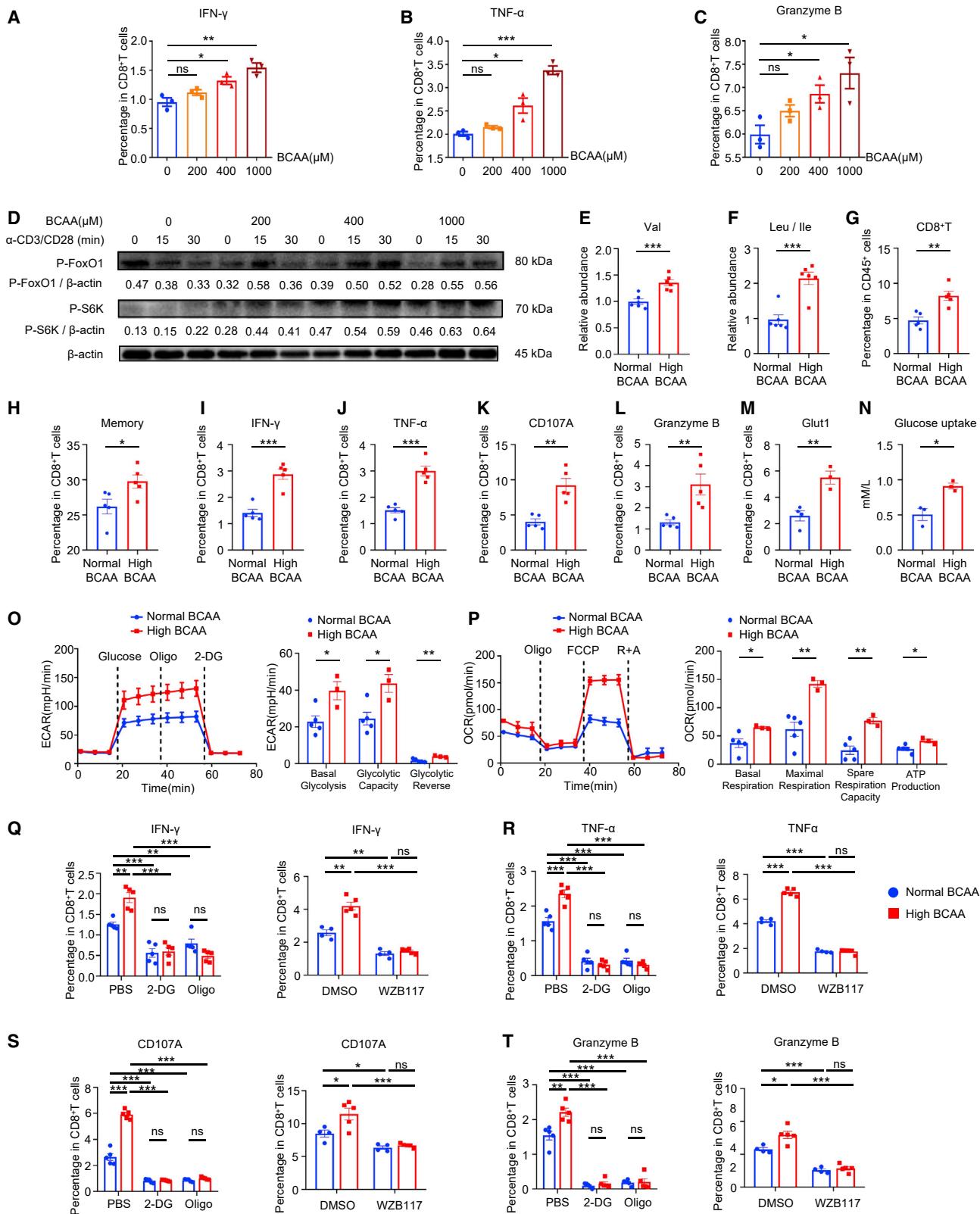
BCAA supplementation upregulates glycolysis and OXPHOS activity in CD8⁺ T cells with functional enhancement

Aforementioned results from *PP2Cm*^{-/-} mice strongly imply that accumulation of intracellular BCAs promotes CD8⁺ T cell functionality largely through modulating glucose metabolism. Whether external supplementation of BCAs recapitulates *PP2Cm* deficiency mediated BCAA accumulation leading to the enhancement of CD8⁺ T cell functions is further investigated. First, we stimulated the splenocytes from WT mice with anti-CD3/CD28 antibodies in the presence of gradient concentrations of BCAs *in vitro*. We found that the percentages of IFN-

γ and TNF- α producing CD8⁺ T cells as well GzmB⁺CD8⁺ T cells increased significantly in a dose-dependent manner (Figures 4A–4C). The percentages of CD44⁺CD8⁺ T cells and the cytotoxicity of CD8⁺ T cells to LLC cells *in vitro* were elevated at high BCAA concentrations as well (Figure S5A and S5B). Since PP2Cm is the key phosphatase to regulate the catabolism of BCAs, we further used a PP2Cm inhibitor Sanguinarine chloride (SC) to analyze the roles of PP2Cm enzymatic activity in modulating CD8⁺ T cell functionality. It was apparent that addition of SC led to the over-activation of CD8⁺ T cells with increased percentages of IFN- γ ⁺, TNF- α ⁺, GzmB⁺, and CD107A⁺ CD8⁺ T cells when compared with DMSO treatment. When BCAs were free in the culture medium, the enhancement of CD8⁺ T cell functionality was suppressed even with the addition of SC (Figures S5C–S5F). Moreover, the addition of BCAs resulted in an increase in phospho-S6K and phospho-FoxO1 (Figure 4D) in CD8⁺ T cells upon *in vitro* activation. When we fed the mice with extra BCAs in water (Figure S5H), BCAA-supplemented mice (indicated as High BCAA) possessed higher levels of plasma BCAs than non-supplemented mice (indicated as Normal BCAA) (Figures 4E and 4F). The abundance of total and memory CD8⁺ T cells in the spleens from the High BCAA group was increased compared with those in the Normal BCAA group (Figures 4G and 4H), whereas the percentages of CD4⁺ T cells, B cells, and NK cells were similar in both groups (Figure S5I). The expression of TCF-7 in CD8⁺ T cells was also significantly upregulated in the High BCAA group when compared with the Normal BCAA group (Figure S5J). Similar to *PP2Cm*^{-/-} mice, High BCAA group mice showed the increases in IFN- γ and TNF- α production by CD8⁺ T cells (Figures 4I and 4J), and the proportions of CD107A⁺ and GzmB⁺ CD8⁺ T cells (Figures 4K and 4L) as well as tumor-killing capacity of CD8⁺ T cells (Figure S5K) as compared with Normal BCAA group mice. When we adoptively transferred CD8⁺ T cells from normal and high BCAA-fed WT mice into *Rag1*^{-/-} mice and detected LLC tumor growth *in vivo*, it was found that tumor volumes in *Rag1*^{-/-} mice containing CD8⁺ T cells from high BCAA-fed mice were smaller than those from normal BCAA-fed mice (Figure S5L). CD8⁺ T cells from the High BCAA group also exhibited increased phospho-PI3K, phospho-S6K, phospho-AKT^{S473}, and phospho-FoxO1 (Figure S5M). Inhibition of FoxO1 activity diminished the increase in cytokine production (Figures S5N and S5O) and the cytotoxicity (Figures S5P and S5Q) in activated CD8⁺

Figure 3. *PP2Cm* deficiency promotes glycolysis and OXPHOS activities in CD8⁺ T cells

- (A and B) Heatmaps showing relative expression of the genes involved in glycolysis and OXPHOS in CD8⁺ T cells from WT (n = 2) versus *PP2Cm*^{-/-} mice (n = 2).
- (C) ECAR influxes of CD8⁺ T cells from WT (n = 3) versus *PP2Cm*^{-/-} mice (n = 3). Seahorse assay was performed with activated CD8⁺ T cells.
- (D) OCR influxes of CD8⁺ T cells from WT (n = 3) versus *PP2Cm*^{-/-} mice (n = 3).
- (E and F) Detection of Glut1 expression on CD8⁺ T cells from WT (n = 5) and *PP2Cm*^{-/-} (n = 4) mice by qPCR with fold changes (E) and by flow cytometry indicated by mean fluorescence intensity (MFI) (F).
- (G) Glucose uptake by CD8⁺ T cells (G) from WT (n = 5) versus *PP2Cm*^{-/-} (n = 4) mice. Glucose concentration in the supernatant was measured after anti-CD3/CD28 stimulation.
- (H) Heatmap showing the ratio of the concentrations of intracellular glycolysis and TCA cycle intermediates in activated CD8⁺ T cells from WT (n = 3) versus *PP2Cm*^{-/-} (n = 3) mice.
- (I) Mass isotopomer distribution (MID) of cellular metabolites in CD8⁺ T cells upon anti-CD3/CD28 stimulation for 24 h. The incorporation of ¹³C atoms from glucose are denoted as M + n, where n is the number of ¹³C atoms and M means mass isotopomer.
- (J–M) Percentages of IFN- γ ⁺ (J), TNF- α ⁺ (K), CD107A⁺ (L), and GzmB⁺ (M) CD8⁺ T cells upon treatment with PBS, 2-DG, and Oligomycin or DMSO versus WZB117. Data are presented as mean \pm SEM. *p < 0.05, **p < 0.01, ***p < 0.001 by Student's t test (C–M). Data are representative of two independent experiments.



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T cells from High BCAA group mice when compared with Normal BCAA group mice. These results demonstrate that BCAA supplementation promotes the activation and effector function of CD8⁺ T cells both *in vitro* and *in vivo*.

Since Glut1 expression was upregulated on CD8⁺ T cells from High BCAA group mice (Figure 4M) together with the increased capability of glucose uptake (Figure 4N), which was relying on FoxO1 activity (Figure S5R) as well as PP2Cm enzymatic activity (Figure S5G), we examined the metabolic consequence of BCAA supplementation. CD8⁺ T cells from High BCAA group mice showed significantly increased ECAR (Figure 4O) and OCR (Figure 4P) influxes when compared with those from Normal BCAA group mice. Intracellular Val and Ile/Leu were accumulated and the concentrations of intracellular glycolytic and TCA cycle intermediates were increased in CD8⁺ T cells from High BCAA group mice according to the metabolomic analysis (Figure S6A). Treatment of CD8⁺ T cells from High BCAA group mice with WZB117, 2-DG, or Oligo reduced their cytokine production (Figures 4Q and 4R) and tumor-killing capacity (Figures 4S and 4T) to the base levels in Normal BCAA group mice. WZB117 treatment also downregulated the abundance of glycolysis and TCA cycle intermediates in CD8⁺ T cells from High BCAA group mice (Figures S6B–S6F). In addition, WZB117 treatment also downregulated TCF-7 expression (Figure S6G) and upregulated Tim-3 and PD-1 (Figures S6H and S6I). Therefore, BCAA supplementation leads to the accumulation of BCAs inside CD8⁺ T cells with hyper-function through the upregulation of Glut1 and increased glycolysis and OXPHOS activities.

BCAA supplementation synergizes with anti-PD-1 regimen in anti-tumor immunity

Given that accumulated intracellular BCAs promote the activation and effector functions of CD8⁺ T cells, we sought to determine whether BCAA supplementation could enhance anti-tumor efficacy either alone or with the combination of programmed cell death protein 1 (PD-1) blockade therapy in mice harboring LLC or B16-F10 tumors (Figure 5A). There was no dramatic difference in tumor growth and tumor weight of LLC and B16-F10 implanted tumors between High and Normal BCAA group mice (Figures 5B and 5C, blue and red). However, the addition of anti-PD-1 Ab significantly slowed down tumor growth with less tumor weight in High BCAA group mice (Figures 5B and 5C, orange lines and dots). When defining the properties of tumor-infiltrating CD8⁺ T cells in High and Normal BCAA group mice with or without anti-PD-1 injection, we found that infiltrating CD8⁺ T cells contained more IFN- γ and TNF- α producing (Figures 5D and 5E),

and CD107A and GzmB-positive proportions (Figures 5F and 5G) in LLC and B16-F10-grafted tumors (Figures S7A–S7D). Infiltrating CD8⁺ T cells from High BCAs + anti-PD-1 Ab-treated tumors exhibited the lowest expressions of TOX (Figure S7E) and the highest expressions of TCF-7 and CXCR5 (Figures S7F and S7G) among four groups. These results thus reveal that although high BCAA content alone does not suppress tumor growth, the addition of anti-PD-1 intervention is likely to trigger infiltrating CD8⁺ T cells with more functional signatures, which in turn amplifies anti-tumoral response to restrain tumor growth.

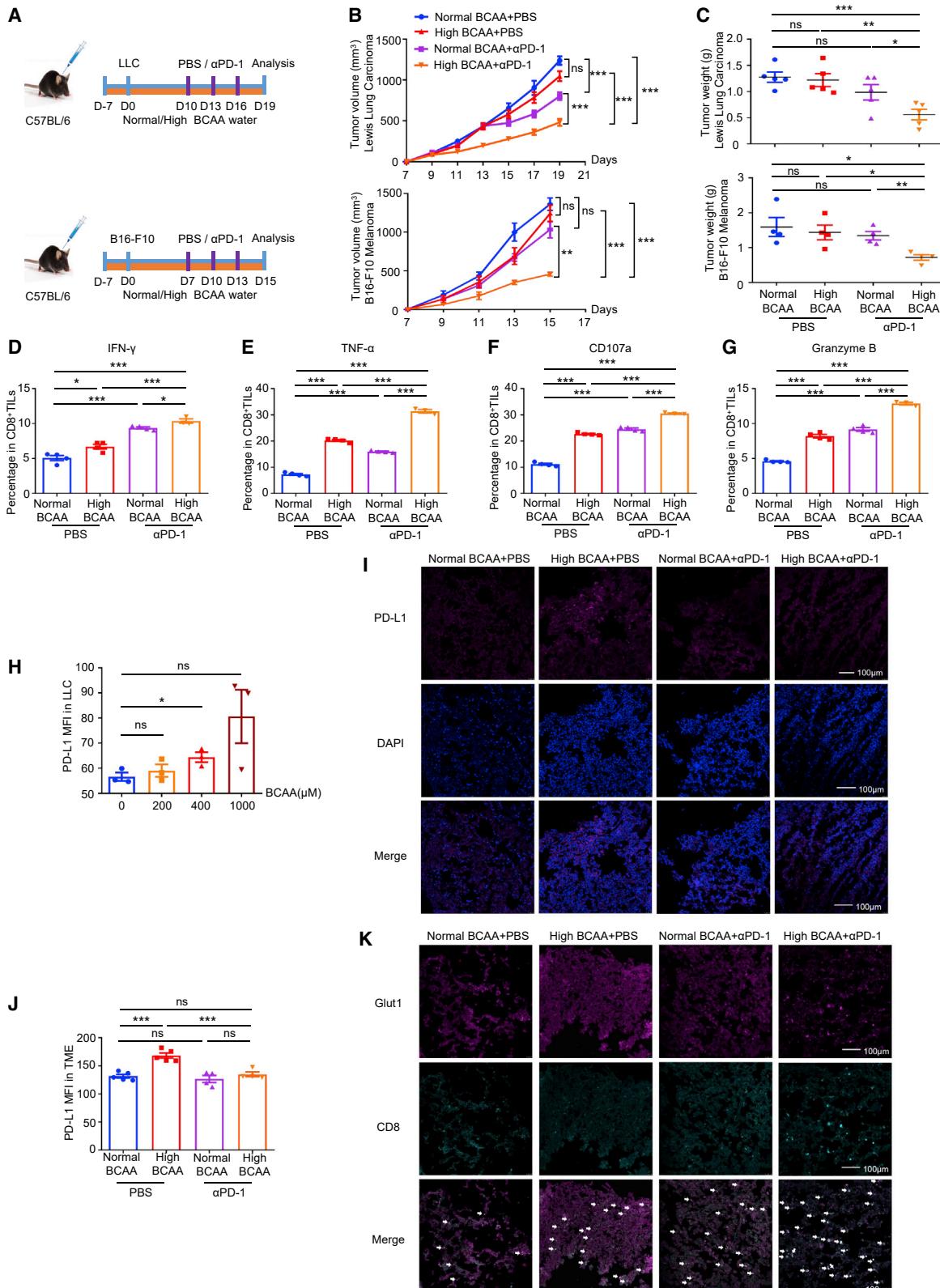
Previous studies have reported the roles of BCAs in tumorigenesis.³⁵ We also observed the effects of BCAs in promoting the proliferation of LLC and B16-F10 *in vitro* in a dose-dependent manner significantly (Figure S7H). Interestingly, when coculturing WT splenocytes with LLC tumor cells *in vitro* at different concentrations of BCAs, we found that the expression levels of PD-L1 on LLC cells were increased significantly at high BCAA concentrations (Figure 5H). In the *in vivo* models, PD-L1 expressions were also higher in LLC-implanted tumors from High BCAA group mice when compared with those from Normal BCAA group mice (Figure 5I). With the additional injection of anti-PD-1 Ab, there displayed downregulation of PD-L1 levels in tumor regions from High BCAA + anti-PD-1 group mice with more significance (Figure 5J). Since high BCAs induced Glut1 expression on CD8⁺ T cells leading to more glucose uptake (Figures 4M and 4N), we also compared Glut1 expressions on infiltrating CD8⁺ T cells among four groups. It was found that LLC grafted tumors from High BCAA group mice displayed increased Glut1 expression on infiltrating CD8⁺ T cells when compared with those from Normal BCAA group mice no matter with or without anti-PD-1 treatment (Figure 5K). Considering the sequential treatment of BCAs (early) and anti-PD-1 Ab (late) in our experimental models, our data strongly imply that BCAA supplementation upregulates Glut1 expression on CD8⁺ T cells, which in turn facilitates infiltrating CD8⁺ T cells to uptake more glucose and synergize with anti-PD-1 in regional anti-tumor immunity.

High BCAs promote the activation of human CD8⁺ T cells and are associated with improved clinical outcomes of anti-PD-1 therapy in non-small cell lung cancer

Our data from mouse models supported that BCAA accumulation either with intrinsic catabolism defect (PP2Cm deficiency) or external supplementation favored CD8⁺ T cell hyper-functionality and synergized with anti-PD-1 therapy in anti-tumor

Figure 4. BCAA supplementation upregulates glycolysis and OXPHOS activity in CD8⁺ T cells with functional enhancement

(A–C) Percentages of IFN- γ ⁺ (A), TNF- α ⁺ (B) and GzmB⁺ (C) CD8⁺ T cells from WT mice ($n = 3$) treated with gradient BCAs *in vitro*. (D) Immunoblotting analysis of the indicated proteins in CD8⁺ T cells from WT mice upon anti-CD3/28 stimulation with gradient BCAA concentrations *in vitro*. (E and F) Levels of intracellular Val (left) and Leu/Ile (right) in mice fed with normal ($n = 6$) versus high BCAA ($n = 6$) water. (G–L) Frequencies of total CD8⁺ T cells (G) and percentages of CD44⁺ (H), IFN- γ ⁺ (I), TNF- α ⁺ (J), CD107A⁺ (K) and GzmB⁺ (L) CD8⁺ T cells in the spleens from the mice fed with normal ($n = 5$) versus high BCAA ($n = 5$) water. (M and N) Glut1 expression levels (M) and glucose uptake (N) in CD8⁺ T cells from the mice fed with normal ($n = 5$) versus high BCAA ($n = 5$) water. (O) ECAR influxes of CD8⁺ T cells from the mice fed with normal ($n = 3$) versus high BCAA ($n = 3$) water. Seahorse assay was performed with activated CD8⁺ T cells. (P) OCR influxes of CD8⁺ T cells from the mice fed with normal ($n = 3$) versus high BCAA ($n = 3$) water. (Q–T) Percentages of IFN- γ ⁺ (Q), TNF- α ⁺ (R), CD107A⁺ (S), and GzmB⁺ (T) CD8⁺ T cells from the mice fed with normal ($n = 3$) versus high BCAA ($n = 3$) water upon treatment with PBS, 2-DG or Oligomycin or DMSO versus WZB117. Data are presented as mean \pm SEM. ns, non-significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Student's t test (A–C, E–T). Data are representative of two independent experiments.



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immunity. We further determined whether increased BCAAs also promoted the activation and function of human CD8⁺ T cells. Peripheral blood mononuclear cells (PBMCs) were freshly isolated from healthy donors and activated by anti-CD3/CD28 *in vitro* in the presence of increasing concentrations of BCAAs. We found that BCAA supplementation increased the populations of CD8⁺ T cells expressing TNF- α and GzmB (Figures 6A and 6B) together with the upregulation of Glut1 expression along with increased BCAA concentrations (Figure 6C).

To assess the clinical significance of our findings, we determined serum BCAA levels in 24 patients with advanced non-small cell lung cancer (NSCLC) (Table S1) receiving nivolumab (an anti-PD-1 therapeutic antibody) treatment. Among them, 10 patients being evaluated as a partial response or stable disease at the first evaluation time were classified as the responders, and 14 with disease progression were classified as the non-responders. We found that the responders had significantly higher levels of Val and Leu/Ile than the non-responders after the first infusion of nivolumab (Figure 6D). High levels of serum Val and Leu/Ile were associated with improved overall survival (Val: hazard ratio [HR] = 0.2350; 95% confidence interval [CI], 0.070–0.792; p = 0.0185; Leu/Ile: HR = 0.2338; 95% CI, 0.0690–0.788; p = 0.0179) (Figure 6E). The abundance of free Leu/Ile was positively correlated with serum IFN- γ and the percentages of peripheral central memory CD8⁺ T cells (Figure 6F) in our patient cohorts. Therefore, high BCAAs also promote human CD8⁺ T cell functionality and might be favorable to improve anti-PD-1 treatment in clinic.

DISCUSSION

In the present study, we have demonstrated that accumulation of BCAAs either due to the impairment of BCAA catabolism with *PP2Cm* deficiency or through diet supplementation could induce CD8⁺ T cell hyper-functionality largely through the upregulation of Glut1 and remodeling intracellular metabolic activity. Previous studies reported that the lack of dietary BCAAs impaired antibody production and the generation of cytotoxic T cells in response to *Salmonella typhimurium*.³⁶ On the contrary high BCAAs promote oxidative stress and inflammation of human PBMCs through mTORC1 activation.³⁷ In our study, BCAA accumulation became a triggering factor for hyper-functionality of CD8⁺ T cells upon TCR/CD28 stimulation. When *PP2Cm*^{-/-} mice were activated in low BCAA culture medium, the percentages of IFN- γ ⁺, TNF- α ⁺, GzmB⁺, and CD107A⁺ CD8⁺ T cells

were downregulated to the similar levels in WT mice (Figures S2P–S2S). The increased metabolism and functionality were attenuated as well (Figures S2T–S2Z). These results further support the requirement of high BCAAs in improving T cell functionality. What is more, Glut1 expression as well as glucose uptake are augmented, which largely contributes to the enhanced ECAR and OCR influxes in CD8⁺ T cells from two BCAA-accumulated mouse models. It has been reported previously that BCAAs were able to regulate the expression of glucose transporter and glucose uptake in skeletal muscle.³⁸ Glut1 expression on CD8⁺ T cells can also be triggered by TCR/CD28 stimulation and modulated by multiplex factors.³⁹ Since glucose is the primary energy resource for T cell activation, clonal expansion, and effector function,⁴⁰ upregulation of Glut1 and subsequent glucose uptake by CD8⁺ T cells under high BCAA content will favor the activation and maintenance of effector function in CD8⁺ T cells as well as the suppression of exhaustion (Figures S6G–S6I). Moreover, results from both gene signature profiles and metabolomic analyses on *PP2Cm*^{-/-} CD8⁺ T cells reveal the reprogramming of glucose metabolism in CD8⁺ T cells (Figure S4). In fact, high activity of both glycolysis and OXPHOS in CD8⁺ T cells was also reported previously in *sirt2*^{-/-} mice where *sirt2* deficiency provoked hyper-acetylation of multiple metabolic enzymes and amplification of their activities.⁴⁰ Herein we have also observed the upregulation of multiplex genes related to energy metabolism, supporting the roles of intrinsic high BCAA content in promoting gene expression through PI3K-AKT-Foxo1 signaling pathways. Interestingly, we did not enrich fatty acid metabolism-related pathway in *PP2Cm*^{-/-} CD8⁺ T cells (Figures S3K, p = 0.6712) with no difference in expression levels of relevant genes between WT and *PP2Cm*^{-/-} CD8⁺ T cells including *Cpt1a*, *ACAA2*, *ECHS1*, *ACSS1*, and *ACSS2* (Figures S3L–S3P). This might be because high BCAAs are more likely to serve as nutrient signal molecules to activate mTOR and other signaling pathways, and integrate multiple metabolic pathways to maintain memory-like phenotypes of CD8⁺ T cells. Glut1 upregulation is one of the outcomes of accumulated BCAAs-triggered gene transcription, which in turn exaggerates glucose consumption and downstream metabolism to fulfill the requirement for the hyper-activity of CD8⁺ T cells.

The fact that upregulation of Glut1 and increased glucose uptake dedicate to enhanced CD8⁺ T cell functionality in high BCAA content might be more significant when CD8⁺ T cells are infiltrating into tumor regions. The TME can be considered as a

Figure 5. BCAA supplementation synergizes with anti-PD-1 regimen in anti-tumor immunity

- (A) Experimental scheme.
- (B and C) Tumor growth (B) and tumor weight (C) of LLC (upper, n = 5 in each group) and B16-F10 (down, n = 4 for each group) transplanted tumors in the mice fed with normal versus high BCAA water with or without anti-PD1 antibody.
- (D–G) Percentages of IFN- γ ⁺ (D), TNF- α ⁺ (E), CD107A⁺ (F), and GzmB⁺ (G) CD8⁺ T cells in LLC grafted tumors (n = 4 mice in each group) from the mice fed with normal or high BCAA water with or without anti-PD1 antibody.
- (H) MFI values of PD-L1 on LLC cells after coculturing with WT CD8⁺ T cells after anti-CD3/CD28 stimulation in gradient BCAA concentrations *in vitro*.
- (I and J) Immunofluorescence staining (I) and MFI values (J) of PD-L1 in LLC grafted tumors from the mice fed with normal versus high BCAA water with or without anti-PD1 antibody (n = 5 in PBS group, n = 4 in anti-PD-1 group). Purple (Cy3) indicates the PD-L1. Blue (DAPI) indicates the nucleus. Scale bar, 100 μ m (\times 200).
- (K) Immunofluorescence staining of CD8 and Glut1 in LLC grafted tumors from the mice fed with normal versus high BCAA water with or without anti-PD1 antibody. Green (FITC) indicates CD8 and purple (Cy3) indicates Glut1. Blue (DAPI) indicates the nucleus. White arrows show co-location of CD8 and Glut1. Scale bar, 100 μ m (\times 200). Data are presented as mean \pm SEM. ns, non-significant; *p < 0.05, **p < 0.01, ***p < 0.001 by Student's t test. Data are representative of two independent experiments.

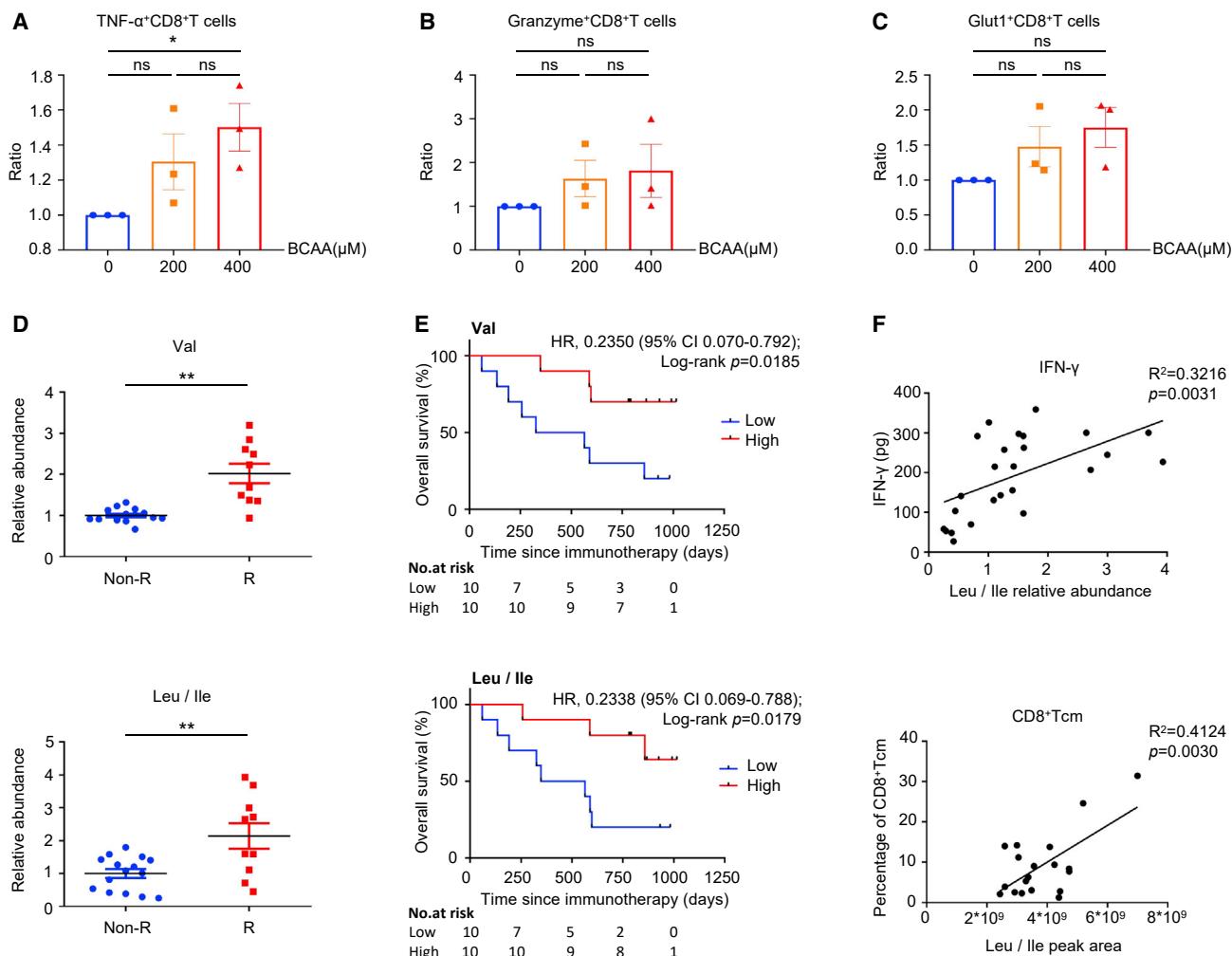


Figure 6. High BCAAs promote human CD8⁺ T cell functionality and are associated with a better prognosis of anti-PD-1 immunotherapy in non-small cell lung cancer

(A-C) Percentages of TNF-α⁺ (A), GzmB⁺ (B), and Glut1⁺ (C) CD8⁺ T cells in PBMCs from healthy donors treated with gradient BCAA concentrations. Samples are normalized to BCAA free group.

(D) Val (upper) and Leu/Ile (lower) levels in the plasma of the responder versus the non-responder NSCLC patients.

(E) Kaplan-Meier (K-M) plots of overall survival (OS) with plasma Val (upper) and Leu/Ile (lower) of NSCLC patients after receiving nivolumab treatment.

(F) Spearman correlations of plasma Leu/Ile abundance with peripheral IFN-γ levels (upper) and the percentages of peripheral central memory CD8⁺ T cells (lower). Data are presented as mean ± SEM; **p < 0.01 by Student's t test (A-D). Survival curves for the PFS were estimated by using the Kaplan-Meier method (the log rank test). The Spearman correlation test was applied to define the associations between BCAA concentrations and immune parameters.

unique metabolic niche that can reshape biological properties of tumor cells, immune cells, and stromal cells inside.⁴¹ A hallmark feature of tumor cell metabolism is increased nutrient consumption to meet energetic, anabolic, and pro-survival demands.⁴² Tumor cells consume massive nutrition in priority for their proliferation leading to the generation of hypoxia, hypoglycemic, and acidic microenvironment, which makes local metabolic landscapes in ways that inhibit anti-tumor immunity.⁴³ To some extent, tumor cells in the TME establish a metabolic barrier for intratumoral CD8⁺ T cells to exert normal anti-tumor immunity.⁴⁴ Based on our results, accumulation of BCAAs either due to PP2Cm knockdown or through external supplementation endows CD8⁺ T cells with the capacity to increase the uptake of

glucose through upregulation of Glut1 expression, which can largely promote T cell effector function in the TME. In the TME, this feature will be of more significance in facilitating CD8⁺ T cells to compete with tumor cells for the utility of glucose, which in turn exaggerates energetic metabolism for the functional requirement. This pattern is mirrored by tumor-infiltrating CD8⁺ T cells from BCAA-supplemented mice, indicating that metabolically stressed CD8⁺ T cells inside tumors under a normal diet might be relieved by BCAA supplementation with the activation of Glut1-mediated glucose competition (Figure 5K).

Our study also provides new evidence on dietary perturbations for combination immunotherapy in tumor control. Previous

investigations demonstrated the beneficial roles of the diet on anti-tumor immunity. For instance, supplementations with inosine,⁴⁵ acetate,⁴⁶ or polyunsaturated fatty acids⁴⁷ under certain circumstances increase anti-tumor immunity. In this study we could not ignore the effects of BCAAs on tumor cells during BCAA supplementation. BCAAs are critical to providing carbon and nitrogen for macromolecule synthesis for tumor growth.⁴⁸ Several studies have reported tumorigenesis effects of BCAA metabolism on tumor cells. In our *in vivo* study, tumor growth was comparable with or without BCAA supplementation. Although we have also observed that BCAAs promoted tumor cell proliferation *in vitro* (Figure S7H), high BCAAs are able to increase CD8⁺ T cell functionality, which in turn restrains tumor growth *in vivo*. We deduce that an extra BCAA diet may amplify the metabolic tug of war in tumors with more effects on the augmentation of local CD8⁺ T cell functionality through the upregulation of glucose transporters. The addition of high BCAAs not only upregulates PD-L1 expression on tumor cells both *in vitro* and *in vivo* (Figures 5H–5J), but also enhances CD8⁺ T cell functionality, which coordinates to favor clinical efficacy of immune checkpoint inhibitor (ICI) treatment. This is pretty well matched with clinical observations that high BCAA levels were favorable for anti-PD-1 reagent treatment. These correlation results together with BCAA supplementation experiments in the mice suggest that high BCAA-mediated metabolic adaptation of tumor-infiltrating CD8⁺ T cells might be one of the key switches to increased anti-tumor immunity, which needs to be functionally confirmed in the future.

In summary, we report that accumulation of BCAAs induces metabolic rewiring of CD8⁺ T cells, which in turn enhances their effector function and anti-tumor immunity. High BCAAs also work in synergy with PD-1 blockade in immunotherapy. With the help of extra BCAAs, metabolic reprogramming initiated from increased glucose uptake facilitates CD8⁺ T cells with more glucose availability to compete with tumor cells in the TME and overcome the potential energy shortage, which fulfills enhanced anti-tumor immune responses locally. Since metabolic states of immune cells and tumor cells as well as other stroma cells within the tumors are coupled, our findings also provide new ways to remodel metabolic reciprocity in intratumoral immune cell populations for tumor control through BCAA supplementation. Once we understand more extensively the effects of BCAA supplementation on the interplay between systemic metabolism and the changes in the TME as well as the effects on the metabolisms of tumor and immune cells, nutrient manipulation with BCAA supplementation might be introduced as a potential combination therapy with ICIs in clinic.

Limitations of the study

In this study, we have proposed that the accumulation of BCAAs reprogrammed glucose metabolism of tumor-infiltrating CD8⁺ T cells, which was dedicated to enhanced anti-tumor immunity through Glut1 hyper-expression. It will be more evident if we could define the metabolic property of tumor infiltrating CD8⁺ T cells as well as tumor cells in the TME to elucidate the roles of nutrition competition in anti-tumor immunity. Since BCAAs have been included in nutrient supply in clinic, we did not validate synergistic roles of BCAAs with anti-PD-1 regimen in clinic.

which could provide clinical evidence on BCAAs for a better prognosis of advanced NSCLC patients receiving ICI treatment.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2023.112186>.

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AUTHOR CONTRIBUTIONS

Y.W., S.L., and H.P.S. initiated the study. Y.W. and C.C.Y. designed the project and directed the research; C.C.Y., R.M.S., and R.C. performed most of the experiment. H.Y.Z. and C.Y. performed the metabolic experiment and data analysis. S.L., Y.Y., W.L.M.Z., and L.L.X. were responsible for sample collection and data analysis. Y.W., C.C.Y., R.M.S., H.P.S., C.Y., and S.L. analyzed the data and drafted the manuscript. All authors contributed to the manuscript preparation.

DECLARATION OF INTERESTS

The authors declare no competing interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Fixable viability stain 510	BD Biosciences	Cat# 564406; RRID: AB_2869572
APC-Cy™7 Rat Anti-Mouse CD45 (30-F11)	BD Biosciences	Cat# 557659; RRID: AB_396774
BV711 Hamster Anti-Mouse CD3e (145-2C11)	BD Biosciences	Cat# 563123; RRID: AB_2687954
BV650 Rat Anti-mouse CD8a (53-6.7)	Biolegend	Cat# 100742; RRID: AB_2563056
PerCP-Cy™5.5 Rat Anti-Mouse CD45R/B220 (RA3-6B2)	BD Biosciences	Cat# 552771; RRID: AB_394457
FITC Mouse Anti-Mouse NK1.1(PK136)	eBioscience	Cat# 11-5941-82; RRID: AB_465318
FITC Rat Anti-Mouse CD44 (IM7)	BD Biosciences	Cat# Cat# 553133; RRID: AB_2076224
APC-R700 Rat Anti-Mouse CD62L (MEL-14)	BD Biosciences	Cat# 565159; RRID: AB_2737397
FITC Rat Anti-Mouse IFN-γ (XMG1.2)	BD Biosciences	Cat# 554411; RRID: AB_395375
PE-Cyanine7 Rat Anti-Mouse TNF alpha (MP6-XT22) (MP6-XT22)	eBioscience	Cat# 25-7321-82; RRID: AB_11042728
FITC Rat Anti-Mouse CD107a (1D4B)	BD Biosciences	Cat# 553793; RRID: AB_395057
PE Rat Anti-Mouse Granzyme B (NGZB)	Invitrogen	Cat# 12-8898-82; RRID: AB_10870787
BV786 Mouse Anti-Mouse CD366 (TIM-3) (7D3)	BD Biosciences	Cat# 747621; RRID: AB_2744187
PE Rat Anti-Mouse TOX (TXRX10)	Invitrogen	Cat# 12-6502-82; RRID: AB_10855034
BV421 Mouse Anti-TCF-7/TCF-1 (S33-966)	BD Biosciences	Cat# 566692; RRID: AB_2869822
PE/Cyanine7 Rat Anti-mouse CD185 (CXCR5) (L138D7)	Biolegend	Cat# 145516; RRID: AB_2562210
Alexa Fluor® 647 Rabbit Anti-Mouse GLUT1 (EPR3915)	Abcam	Cat# ab195020; RRID: AB_2783877
APC Hamster Anti-Mouse CD279 (PD-1) (J43)	BD Biosciences	Cat# 562671; RRID: AB_2737712
BV421 Hamster Anti-Mouse CD279 (PD-1) (J43)	BD Biosciences	Cat# 562584; RRID: AB_2737668
BV510 Mouse Anti-Human CD3 (HIT3a)	BD Biosciences	Cat# 564713; RRID: AB_2738909
APC Mouse Anti-human CD8a (RPA-T8)	Biolegend	Cat# 301014; RRID: AB_314132
APC/Cyanine7 Mouse Anti-human TNF-α (Mab11)	Biolegend	Cat# 502944; RRID: AB_2562870
FITC Mouse anti-Human Granzyme B (GB11)	BD Biosciences	Cat# 560211; RRID: AB_1645488
PE Mouse Anti-Human Glut1 (202,915)	BD Biosciences	Cat# FAB1418P; RRID: AB_2191040
FITC Mouse Anti-Human CD45RO (UCHL1)	BD Biosciences	Cat# 555492; RRID: AB_395883
APC-H7 Mouse Anti-Human CD27 (M-T271)	BD Biosciences	Cat# 560222; RRID: AB_1645474
InVivoMab anti-mouse PD-1(CD279) (RMP1-14)	BioXCell	Cat# BE0146; RRID: AB_10949053
InVivoMab anti-mouse CD8α (YTS 169.4)	BioXCell	Cat# BE0117; RRID: AB_10950145
Rabbit Anti-Mouse Phospho-p70 S6 Kinase (Thr389) (108D2)	CST	Cat# 9234; RRID: AB_2269803
Rabbit Anti-Mouse β-Actin (13E5)	CST	Cat# 4970; RRID: AB_2223172
Rabbit Anti-Mouse Phospho-Akt (Ser473) (D9E)	CST	Cat# 4060; RRID: AB_2315049
Rabbit Anti-Mouse PI3 Kinase p85 (19H8)	CST	Cat# 4257; RRID: AB_659889
Rabbit Anti-Mouse Phospho-FoxO1 (Ser256)	CST	Cat# 9461; RRID: AB_329831
Mouse PD-L1/B7-H1 Antibody	RD	Cat# AF1019; RRID: AB_354540
Anti-CD8 alpha Rabbit pAb antibody	Servicebio	Cat# GB11068; RRID: AB_2905511
Anti-GLUT1 Rabbit pAb antibody	Servicebio	Cat# GB11066
Cy3 conjugated Donkey Anti-Goat IgG (H + L) antibody	Servicebio	Cat# GB21404; RRID: AB_2868507
Cy3 conjugated Goat Anti-Rabbit IgG antibody	Servicebio	Cat# GB21303; RRID: AB_2861435

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
FITC-labeled goat anti-rabbit IgG antibody	Servicebio	Cat# GB22303; RRID: AB_2904189
Chemicals, peptides, and recombinant proteins		
Phorbol 12-myristate 13-acetate	Sigma-Aldrich	Cat# P1585
Ionomycin calcium salt	Sigma-Aldrich	Cat# I3909
Fixation/Permeabilization Solution		N/A
Kit with BD GolgiStop™	BD Biosciences	Cat# 554715
Transcription Factor Buffer Set	BD Biosciences	Cat# 562574
Leucine	Sigma-Aldrich	Cat# L8000
Valine	Sigma-Aldrich	Cat# V5013
Isoleucine	Sigma-Aldrich	Cat# I2752
2-Deoxy-D-glucose	Sigma-Aldrich	Cat# D6134
Oligomycin	Sigma-Aldrich	Cat# O4876
AS1842856	Selleck	Cat# S8222
Sanguinarine chloride	Selleck	Cat# S5452
WZB117	Selleck	Cat# S7927
Rapamycin	Selleck	Cat# S1039
Lymphoprep	Serumwerk Bernburg AG	Cat# 1858
Mouse Recombinant IL-2	Stemcell	Cat# 78081
Foundation Fetal Bovine Serum	Gemini	Cat# 900-108
Dulbecco's Modified Eagle Medium (DMEM)	HyClone	Cat# SH30243.01
RPMI 1640 medium	HyClone	Cat# SH30809.01
Phosphate Buffered Saline	HyClone	Cat# SH30256.01
Dulbecco's Modified Eagle Medium (DMEM) without D-Glucose	Thermo Fisher Scientific	Cat# 11966025
RPMI 1640 medium without L-leucine, L-valine, L-isoleucine	BasalMedia	Cat# X054E1
Penicillin/Streptomycin	Thermo Fisher Scientific	Cat# 15140122
L-Glutamine	Thermo Fisher Scientific	Cat# 25030081
HEPES Buffer Solution	Thermo Fisher Scientific	Cat# 15630080
MEM Non-Essential Amino Acids Solution	Thermo Fisher Scientific	Cat# 11140050
2-mercaptoethanol	Thermo Fisher Scientific	Cat# 21985023
Universal tissue fixative	Servicebio	Cat# G1101
Sucrose	Sinopharm Group Chemical Reagent Co. LTD	Cat# 57-50-1
OCT embedding agent	Servicebio	Cat# G6059
DAPI	Servicebio	Cat# 1012
RIPA Buffer	Thermo Fisher Scientific	Cat# 89901
PMSF	Thermo Fisher Scientific	Cat# 36978
TRIzol	Thermo Fisher Scientific	Cat# 15596018
PrimeScript™ RT reagent Kit	Takara	Cat# RR037B
TB Green™ Premix Ex Taq™ II	Takara	Cat# RR82WR
Critical commercial assays		
Cytotoxicity LDH Assay Kit-WST	Dojindo	Cat# CK12
XF Glycolytic stress test kit	Agilent Technologies	Cat# 103020-100
XF Cell Mito stress test kit	Agilent Technologies	Cat# 103015-100
EasySep™ Human CD8+T cell Isolation Kit	Stemcell	Cat# 19853A
EasySep™ Human CD8 Positive Selection Kit II	Stemcell	Cat# 17853
Glucose Assay Kit	Abcam	Cat# ab65333

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
L-Lactate Assay Kit (Colorimetric/Fluorometric)	Abcam	Cat# ab65330
Dynabeads™ Mouse T-Activator CD3/CD28	Thermo Fisher Scientific	Cat# 11452D
Dynabeads™ Human T-Activator CD3/CD28	Thermo Fisher Scientific	Cat# 11131D
Tumor Dissociation Kit	MiltenyiBiotec	Cat# 130-096-730
GentleMACS 25 C Tubes	MiltenyiBiotec	Cat# 130-093-237
MACS SmartStrainer	MiltenyiBiotec	Cat# 130-098-462
AllPrep DNA/RNA/miRNA Universal Kit	QIAGEN	Cat# 80224
Dynabeads mRNA DIRECT Micro kit	Invitrogen	Cat# 61021
KAPA Stranded RNA-Seq Library Preparation Kit	KAPA	Cat# KK8400
Protein Assay Kit I	Bio-Rad	Cat# 5000001
Deposited data		
RNA-sequencing data	deposited in Gene Expression Omnibus	GEO: GSE224250
Experimental models: Cell lines		
Mouse B16-F10 melanoma cells	ATCC	CRL-6475, RRID: CVCL_0159
Mouse Lewis lung carcinoma line	ATCC	CRL-1642, RRID: CVCL_4358
Oligonucleotides		
ACTIN-F	AGAAGGACTCCTATGTGGGTGA	N/A
ACTIN-R	CATGAGCTGGGTCATTTCA	N/A
CPT1A-F	CTCCGCCTGAGCCATGAAG	N/A
CPT1A-R	CACCACTGATGATGCCATTCT	N/A
ACAA2-F	CTGCTACGAGGTGTGTCATC	N/A
ACAA2-R	AGCTCTGCATGACATTGCC	N/A
ECHS1-F	TTGTGAACCTGCCATGATGTGT	N/A
ECHS1-R	TGCTCGGGTGAGTCTTGAG	N/A
ACSS1-F	GTTTGGGACACTCCTTACCATAC	N/A
ACSS1-R	AGGCAGTTGACAGACACATT	N/A
ACSS2-F	AAACACGCTCAGGGAAAATCA	N/A
ACSS2-R	ACCGTAGATGTATCCCCCAGG	N/A
Software and algorithms		
FlowJo v.10.CL	FlowJo	https://www.flowjo.com/
Prism v.8.0	GraphPad	http://www.graphpad.com
ImageJ	NIH	https://imagej.nih.gov/ij/
MetaboAnalyst 4.0	N/A	https://www.metaboanalyst.ca/
Other		
Seahorse XFe96 Extracellular Flux Analyzer	Agilent Technologies	N/A
BD LSRFortessa X20	BD Biosciences	N/A
Amersham Imager 600	GE	N/A
Leica Sp8 laser scanning confocal microscope	Leica Biosystems	N/A
Frozen section machine	Thermo Fisher Scientific	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ying Wang (ywangssmu@shsmu.edu.cn).

Materials availability

All materials generated in this study are available from the [lead contact](#).

Data and code availability

- The RNA sequencing dataset generated during this study has been submitted in the Gene Expression Omnibus under the accession number GSE224250.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Human subjects**

Patients enrolled in this study were those with advanced NSCLC participating in two clinical trials (CheckMate 078 [NCT02613507] and CheckMate870 [NCT03195491]) in Shanghai Chest Hospital affiliated to Shanghai Jiao Tong University School of Medicine. Patients enrolled were at stage IIIB/IV with disease progression after one to two prior systemic therapies. All participants had measurable disease severity by computed tomography (CT) or magnetic resonance imaging (MRI) using Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST 1.1). Patients received nivolumab 3 mg/kg intravenously every 2 weeks until disease progression or intolerable toxicity. Responses to the treatment were evaluated every 6 weeks and were confirmed by a subsequent assessment no less than 4 weeks from the date firstly documented. Patients in this study were defined as the responder (R) (with PFS >180 days) or the non-responder (NR) (with PFS <180 days) according to the RECIST 1.1 evaluation. The study was approved by the Ethics Committee of Shanghai Chest Hospital. All patients were informed of the study, consented to the enrollment, and signed an informed consent form. All the procedures were conducted following the Declaration of Helsinki. The basic information of NSCLC patients included in this study summarized in [Table S1](#). Healthy donors were healthy volunteers.

Mice

PP2Cm germ-line knockout (KO) mice with C57BL/6 background were generated as previously described¹³ and maintained in a specific pathogen-free (SPF) animal facility of Shanghai Jiaotong University School of Medicine (SJTUSM). Wild-type (WT) mice used in some experiments were purchased from the Shanghai Laboratory Animal Center (Shanghai, China) and maintained under specific-pathogen-free (SPF) conditions in the animal facility of SJTUSM. Mice were subjected to 12 h light/dark cycles (light cycle from 6 am to 6 pm and dark cycle from 6 pm to 6 am) and were maintained at stable room temperature between 68 and 72°F, with food and water. For all experiments, 6–8-week-old sex-matched mice were used unless otherwise indicated. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University School of Medicine (A-2022-018). Age and sex-matched *Rag1*^{−/−} mice were purchased from The GemPharmatech LLC (Nanjing, China)

Cells lines

The Lewis lung carcinoma cell line (LLC) and B16-F10 melanoma cell line were purchased from the American Type Culture Collection (ATCC) previously and maintained in complete Dulbecco's Modified Eagle Medium (DMEM, ThermoFisher Scientific Inc.) containing 10% fetal bovine serum (FBS, Gemini Inc.) and 1% penicillin/streptomycin (P/S, ThermoFisher Scientific Inc.) at 37°C with 5% CO₂ atmosphere. B16-F10 and LLC cells were used in mouse tumor models, and *in vitro* proliferation and cytotoxicity assay.

METHOD DETAILS**Mouse tumor models**

For tumor models, tumor cells were injected subcutaneously (s.c.) into 8-week-old mice (2×10^5 B16-F10 cells or 5×10^5 LLC cells per mouse). Tumor volumes were monitored from Day 7 and were calculated by using the equation (length x width²)/2. Calculation of tumor volumes was done in a blinded fashion and the lethality was defined as tumor volumes reaching 1500 mm².

For *Rag1*^{−/−} adoptive transfer model, 1×10^6 purified CD8⁺ T cells from the spleens of the mice fed with Normal BCAA or High BCAA water were injected (i.v.) 2 days before LLC tumor inoculation.

***In vivo* anti-PD-1 treatment**

For *in vivo* anti-PD-1 treatment, 200 µg/100 µL purified anti-PD-1 monoclonal antibodies (RMP1-14, BioXcell) were intraperitoneal (i.p.) injected on day 10, 13 and 16 in LLC-implanted mice (5×10^5 LLC cells were injected on Day 0), and on day 7, 10 and 13 in B16-F10-implanted mice (2×10^5 B16-F10 cells were injected on Day 0). Control mice were treated with i.p. injection of 100 µL PBS.

***In vivo* high or low BCAA-fed mouse model**

In high BCAA-fed experiments, mice were fed with water containing 15 mg/mL valine, 15 mg/mL leucine and 15 mg/mL isoleucine throughout the whole experiment while control mice (normal BCAA) were fed with normal sterile ddH₂O. Blood was collected via the Posterior ophthalmic venous plexus for BCAA level measurement. In low BCAA-fed experiments, low BCAA-diet was purchased from XIETONGSHENGWU (Nanjing, China).

In vivo T cell depletion

For *In vivo* depletion of CD8⁺ T cells, 200 µg/100 µL purified anti-CD8 monoclonal antibodies (YTS 169.4, BioXCell) were intraperitoneal (i.p.) injected on day -2, 5 and 12 respectively and 5 × 10⁵ LLC cells or 2 × 10⁵ B16-F10 cells were injected on Day 0. Control mice were treated with *i.p.* injection of 100 µL PBS.

Preparation of mouse tumor infiltrating lymphocytes

Grafted tumors were minced into small pieces and put into Macs C tubes (MiltenyiBiotec) followed by enzymatic digestion in FBS-free DMEM culture medium containing 16 mg/mL Enzyme D, 0.4 mg/mL Enzyme R and 0.5 mg/mL Enzyme A (all from MiltenyiBiotec). Cell suspensions were successively filtered through 70 mm cell strainers (MiltenyiBiotec) to obtain single cell suspension and subjected to further experiments.

Isolation of mouse CD8⁺ T cells and *in vitro* stimulation

Mouse spleens and lymph nodes were homogenized and lymphocytes were prepared by density gradient centrifugation using Lymphoprep (Serumwerk Bernburg AG). CD8⁺ T cells were purified by MACS beads (Stem cell) according to the manufacturer's instructions. The purity was >95% confirmed by flow cytometric analysis. Purified CD8⁺ T cells were stimulated with anti-CD3/CD28 beads (Thermo Fisher Scientific) for 24 h in complete RPMI 1640 medium (Hyclone) supplemented with 10% fetal bovine serum (Gemini), 1% penicillin/streptomycin (P/S, Thermo Fisher Scientific), 1% L-glutamine (Thermo Fisher Scientific), 1% HEPES Buffer Solution (Thermo Fisher Scientific), 1% MEM Non-Essential Amino Acids Solution (Thermo Fisher Scientific) and 0.1% 2-mercaptoethanol (Thermo Fisher Scientific) at 37°C with 5% CO₂. For glucose uptake and lactate production detection, IL-2 (10 ng/mL, Stem Cell) was added additionally during CD8⁺ T cell stimulation. For *in vitro* inhibition experiments, 5 mM 2-DG (Sigma-Aldrich), 10 µM Oligomycin (Sigma-Aldrich), 10 µM WZB117 (Selleck), 1 µM AS1842856 (Selleck), 1 µM Sanguinarine chloride (Selleck) and 100 nM Rapamycin (Selleck) were added during CD8⁺ T cell stimulation. For *in vitro* BCAA supplementation experiment, valine, leucine and isoleucine (all from Sigma-Aldrich) were added in BCAA-free RPMI medium (Basal Media) at the concentration of 10 mM for CD8⁺ T cell stimulation.

Preparation of human CD8⁺ T cells and *in vitro* stimulation

Human peripheral blood mononuclear cells (PBMCs) were prepared by gradient centrifugation using Lymphoprep (Serumwerk Bernburg AG) and washed with RPMI 1640 culture medium. CD8⁺ T cells were purified by MACS beads (StemCell) according to the manufacturer's instructions. The purity was >90% confirmed by flow cytometry. Purified CD8⁺ T cells were stimulated with anti-CD3/CD28 beads (Thermo Fisher Scientific) for 24 h with BCAs at gradient concentrations.

Flow cytometry

For the analysis on surface markers, mouse TILs or splenocytes were stained in PBS containing 2% FBS (FACS buffer) with FVS (BV510) and antibodies targeting mouse CD45 (Apc-cy7), CD3e (BV711), CD8a (BV650), B220 (Percp-cy5.5), NK1.1 (FITC), CD44 (BV785), CD62L (APC-R700), PD-1 (BV421), Tim-3 (BV786) and CXCR5 (PE-cy7), incubated for 35 min at 4°C, washed once with FACS buffer and fixed in PBS containing 1% paraformaldehyde. For human PBMC staining, antibodies targeting CD3 (Bv510), CD8a (APC), CD45RO (FITC) and CD27 (Apc-h7) were used.

For intracellular cytokine detection, cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 100 ng/mL, Sigma-Aldrich), ionomycin (1 mg/mL, Sigma-Aldrich) and GolgiStop™ (0.1%, BD Biosciences) for 4 h. After the stimulation, cells were stained with antibodies targeting surface molecules and subject to intracellular staining by using Cytofix/Cytoperm™ Kit (BD Biosciences) according to the manufacturer's instructions. Briefly, cells were fixed and permeabilized, and followed by adding antibodies targeting IFN-γ (APC-cy7), TNF-α (PE-cy7), CD107A (FITC), GzmB (PE), Glut1 (Alexa 647) for mouse lymphocytes and TNF-α (APC-cy7), GzmB (FITC), Glut1 (PE) for human lymphocytes.

For intracellular transcriptional factor staining, cells were firstly labeled with surface markers before fixation/permeabilization with BD Pharmingen™ Transcription Factor Kit (BD Biosciences) and then stained with antibodies targeting TCF-7 (BV421), TOX (PE), PD-1 (APC) and Tim-3 (BV786).

Cells were acquired on a BD LSR Fortessa X20 (Beckton Dickinson). Data were analyzed with FlowJo Version 10.0 software.

Cell proliferation assay

Cell Counting Kit-8 detection kit (Dojindo) was used to determine the proliferation of tumor cells. In brief, LLC and B16-F10 cells were diluted to the concentration of 30,000 cells/mL. 100 µL cell suspension was added to replicate in 96-well plates and cultured for 0, 24, 48, and 72 h respectively. 10 µL CCK-8 reagent was added to each well followed by incubation for 4 h at 37°C. The absorbance at 450 nm was measured using a PowerWaveXS2 microplate spectrophotometer (BioTek Instruments, Inc., VT, USA).

RNA-sequencing analysis

Purified wild-type (WT) and *PP2Cm*^{-/-} CD8⁺ T cells were stimulated with anti-CD3/CD28 beads for 24 h. Total RNA was extracted using AllPrep DNA/RNA/miRNA Universal Kit (QIAGEN). mRNA was further purified by using Dynabeads mRNA DIRECT Micro kit (Invitrogen) and subjected to library preparation using KAPA Stranded RNA-Seq Library Preparation Kit (KAPA). The libraries were

multiplexed and sequenced on Illumina Nextseq500 (75bp paired-end reads). Raw FASTQ files from RNA-seq assay were aligned to the GRCm38 reference genome using Kallisto (<https://pachterlab.github.io/kallisto/>), filtered to remove transcripts with low counts, and trimmed using the FPKM normalization method. Only transcripts with mean count number >10 were analyzed. To define transcriptional differences between *PP2Cm*^{-/-} and WT CD8⁺ T cells, the intersection of genes of significant differentiation expression (DEGs) from Limma, DESeq2, and EdgeR R packages (R version: 3.8.0) was extracted for volcano plot, and a false discovery rate (FDR) q-value of 0.01 and Log2 fold change of 1 were imposed as the cut-off.

Enrichment analysis

Pathway enrichment was calculated using several biological databases (KEGG, Reactome, Biocarta, and Panther) with an FDR q-value <0.01. Metabolism-related pathways were further enriched according to 61 metabolic genes from the KEGG database as have been indicated in previous reports. For gene set enrichment analysis (GSEA) analysis, GSE9650_EXHAUSTED_VS_MEMORY_CD8_T_cell_DN, GOBP_T_CELL_ACTIVATION, KEGG_OXIDATIVE_PHOSPHORYLATION, HALLMARK_GLYCOLYSIS REACTOME and GOBP_FATTY_ACID_BETA_OXIDATION were performed using GSEA based R package.

Western blot assay

Cells were washed with ice-cold PBS and lysed on ice for 30 min in RIPA buffer (Thermo Fisher Scientific) containing 1mM Phenyl-methanesulfonylfluoride (PMSF) (Thermo Fisher Scientific). Whole-cell extracts were collected by centrifugation and protein concentration was determined by BCA protein assay kit (Bio-Rad Laboratories, CA, USA). Cell lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA) by semi-dry transfer system. The membranes were blocked with PBS (pH7.4) containing 5% non-fat dried milk for 2 h at room temperature and then incubated with primary antibodies overnight at 4°C. After incubation with HRP-conjugated secondary antibodies for 1 h, immunoreactive proteins were visualized with ECL detection system (Millipore).

Immunofluorescence analysis

Fresh tumor tissues were fixed in Universal tissue fixative (Servicebio, Wuhan, China) for more than 24 h. The tissues were removed to 15% sucrose solution (Sinopharm Group Chemical Reagent Co. LTD) at 4°C for 12 h, and 30% sucrose solution at 4°C for 12 h for dehydration. Dehydrated tissues were embedded in the OCT embedding agent (Servicebio) and frozen at -20°C. 8–10 μM sections were sliced on clean slides and subjected to immunofluorescence assay. Briefly, PBS-3% BSA was added to the tissues to block non-specific binding for 30 min and anti-mouse PD-L1 pAb (1:150), anti-mouse CD8α pAb (1:300) or anti-mouse Glut1 pAb (1:300) were incubated with the tissues at 4°C overnight in a wet box. The slides were rinsed three times with PBS (pH 7.4) and incubated with AF488-goat anti-rabbit (1:400), Cy3-donkey anti-Goat (1:300), or Cy3-goat anti-rabbit pAbs (1:300) at room temperature for 50 min in dark. The slides were washed three times with PBS (pH 7.4) for 5 min each. DAPI solution (1:5000) was added for counterstain and mounted using Vectashield fluorescence media. Slides were imaged using Leica Sp8 laser scanning confocal microscope and analyzed by using the ImageJ website online (<https://imagej.nih.gov/ij/>).

Lactate dehydrogenase (LDH) release assay

Cytotoxicity was performed by LDH release assay using CytoTox 96 Non-Radioactive Cytotoxicity assay kit (Dojindo) according to the manufacturer's instructions. Briefly, CD8⁺ T cells were washed with sterile PBS (Hyclone), resuspended in serum-free RPMI 1640 as effector cells at $1 \times 10^7/\text{mL}$, and then mixed with LLC cells ($1 \times 10^5/\text{mL}$) at different effector to target (E: T) ratios. After culturing for 4 h at 37°C, LDH release was detected and cytotoxicity was calculated according to the following formula:

Cytotoxicity (%) = $100 \times (\text{Experimental release} - \text{Target cell spontaneous release}) / (\text{Target cell maximum release} - \text{Target cell spontaneous release})$.

"Experimental release" referred to LDH release from experimental wells. "Target cell spontaneous release" referred to the spontaneous release of LDH from target cells alone. "Target cell maximum release" referred to the maximum release of LDH from target cells in a medium containing 1% Triton X-100 T cells.

Extracellular flux analysis

Glycolysis Stress Test (GST) and mitochondrial stress test (MST) were purchased from Agilent technologies, and Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using the Seahorse XF analyzer (Agilent Technologies) according to the manufacturer's instructions. Briefly, 2×10^5 activated T cells were plated in Cell-Tak (Corning)-coated XF96 plates in RPMI 1640 medium (pH 7.4) containing 2 mM L-glutamine only for GST, or supplemented with 10 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate for GRA and MST (all from Agilent technologies) via centrifugation to ensure cell attachment. Cells were equilibrated for 1 h in a non-CO₂ incubator before starting the assay. For GST, ECAR was measured under basal conditions and in response to 10 mM glucose, 1 μM Oligo, and 50 mM 2-DG successively to calculate the basal glycolytic rate, glycolytic capacity (in response to Oligo), and glycolytic reserve (=glycolytic capacity–basal rate). For MST, OCR was measured under basal conditions and in response to sequential injections of 1 μM Oligo, 1 μM FCCP (Carbonyl cyanide 4-trifluoromethoxyphenyl hydrazone), and 0.5 μM R/A to calculate basal respiration rate (=baseline OCR–R/A OCR), maximal respiration rate (=FCCP OCR–R/A OCR), Spare respiration capacity (=maximal respiration–basal respiration) and ATP production (=Basal Respiration–Proton Leak).

Detection of glucose consumption and lactate production

Freshly isolated CD8⁺ T cells were seeded in 24-well plates (3×10^6 /well) in RPMI 1640 medium and stimulated with anti-CD3/28 beads with the presence of 10 ng/mL IL-2 for 24 h. The supernatants were collected to detect glucose consumption by using Glucose Assay Kit (Abcam). Glucose uptake was calculated as the concentrations of glucose in the medium without the cells minus those in the medium after culturing cells for 24 h. Lactate production was detected by measuring lactate in the supernatants using a lactate Colorimetric/Fluorometric assay kit according to the manufacturer's instructions (Abcam).

Metabolomic analysis

CD8⁺ T cells were activated with anti-CD3/28 beads for 24 h. Cells were washed once with PBS and the metabolites were extracted from freshly activated CD8⁺ T cells using 80% precooled methanol. After 20 min incubation at -80°C, the samples were centrifuged at 15,000 rpm for 10 min at 4°C to remove protein components. The supernatants were collected for target LC-MS/MS detection as described previously. Briefly, cell extracts were analyzed by ultra-high-performance liquid chromatography (Acquity, Waters) coupled to a Q Exactive hybrid quadrupole–orbitrap mass spectrometer (Thermo Fisher). The injection volume was 10 µL. Metabolites were separated with a Luna NH₂ column (100 mm × 2 mm, 3 µm particle size, Phenomenex). The key parameters were as follows: ionization voltage, +3.8 kV/-3.0 kV; sheath gas pressure, 35 arbitrary units; auxiliary gas, 10 arbitrary units; auxiliary gas heater temperature, 350°C; capillary temperature, 320°C. The mass spectrometer was run in full scan mode at an m/z 70–1,000 scan range and 70,000 resolutions. Data processing and ion annotation based on accurate mass in Xcalibur 4.0 (Thermo Fisher). A subset of identified compounds was verified by mass and retention time match to authenticated standards. For the stable-isotope tracing experiment, freshly isolated CD8⁺ T cells were activated with anti-CD3/28 beads in the medium containing 11 mM [U-¹³C]-glucose for 24 h. Cells were washed with PBS once and subjected to LC-MS detection. To determine intermediate metabolites in WZB117 inhibitor assays, CD8⁺ T cells were activated with anti-CD3/28 beads with the presence of 10 µM WZB117 for 24 h. Cells were collected and subjected to target LC-MS/MS detection.

Luminex assay

The concentrations of IFN-γ in the serum were determined using a Luminex assay conducted by LAIZEE BIOTECH CO., LTD including 10 cytokines. Standard curves were generated from the mean fluorescent intensity (MFI) of reference cytokine gradient concentrations by using Luminex200 IS V2.1 Software. The concentrations of IFN-γ in serum samples were calculated according to the standard curves.

RT-PCR

Total RNA of activated CD8⁺ T cells was extracted with TRIzol (Thermo Fisher, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription reactions were conducted using PrimeScript RT reagent Kit (Takara Bio, Tokyo, Japan) according to the manufacturer's instructions. Subsequently, 50 ng of cDNA was used as a qPCR template to quantify genes using TB Green Premix Ex Taq II (Takara Bio, Tokyo, Japan). Target genes related to fatty acid oxidation (the primers were listed in the [key resources table](#)) were amplified over 40 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s. Gene expressions were normalized to β-actin according to the cycle threshold ($2^{-\Delta\Delta CT}$) method.

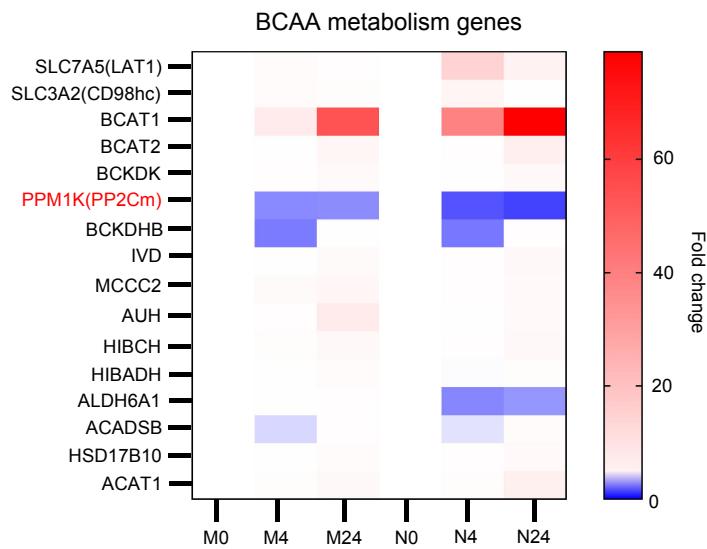
QUANTIFICATION AND STATISTICAL ANALYSIS

Data were presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using Prism 8.0 software. Two-tailed unpaired Student's t tests were performed. Survival curves for the PFS were estimated using the Kaplan-Meier method (log rank test). The Spearman correlation test was applied to perform the association analysis. p < 0.05 was considered statistically significant.

Supplemental information

**Accumulation of branched-chain amino acids
reprograms glucose metabolism in CD8⁺ T cells with
enhanced effector function and anti-tumor response**

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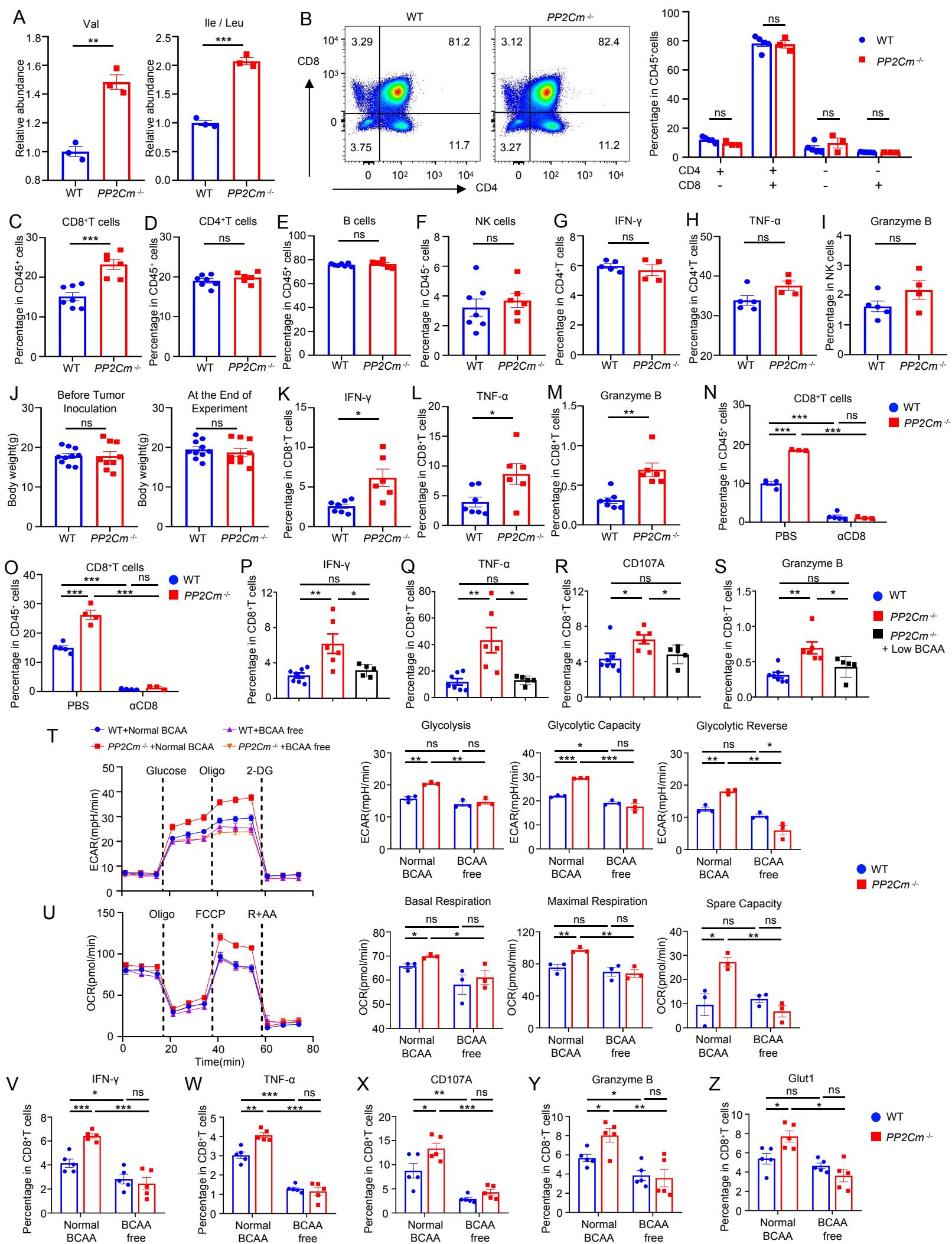


Supplementary Figure 1. Downregulation of *PP2Cm* in human CD8⁺ T cells upon anti-CD3/CD28 activation.

Memory and naive CD8⁺ T cells were purified from peripheral blood mononuclear cells of healthy donors, and were activated by anti-CD3/28 microbeads at the ratio of 1:1 *in vitro* for 4 h or 24 h.

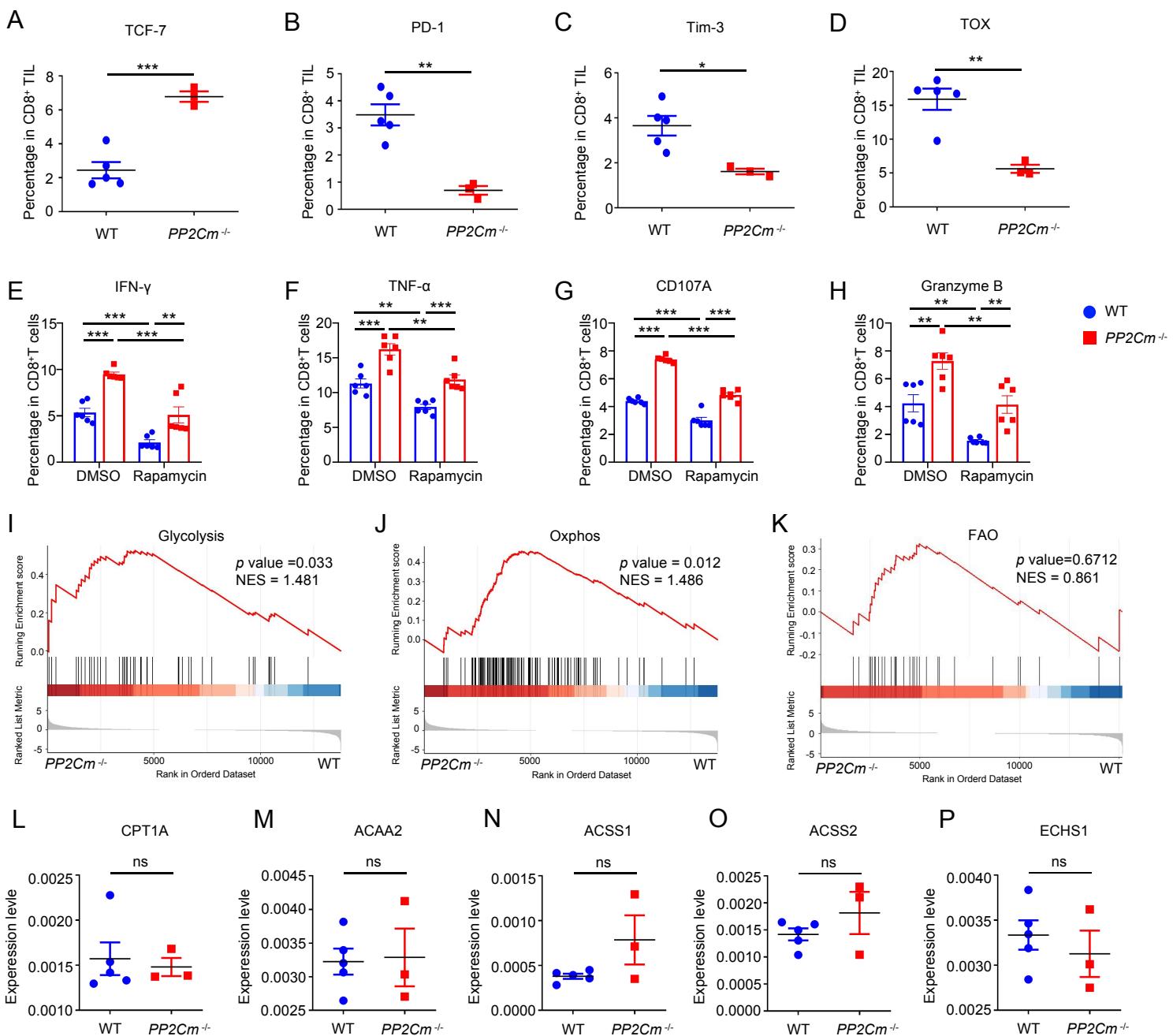
Activated cells were subject to transcriptome analysis by RNA sequencing. Genes related to BCAA metabolism were enriched in which *PP2Cm* was downregulated both in memory and naive CD8⁺ T cells after the activation.

M0: Memory CD8⁺ T cells without activation; M4: Memory CD8⁺ T cells upon anti-CD3/CD28 activation for 4 h; M24: Memory CD8⁺ T cells upon anti-CD3/CD28 activation for 24 h; N0: naive CD8⁺ T cells without activation; N4: Naive CD8⁺ T cells upon anti-CD3/CD28 activation for 4 h; N24: Memory CD8⁺ T cells upon anti-CD3/CD28 activation for 24 h.



Supplementary Figure 2. Related to Figure 1 — *PP2Cm*^{-/-} mice exhibit enhanced CD8+ T cell functionality.

- (A) Comparisons of plasma levels of Val and Leu/Ile in WT (n = 3) versus *PP2Cm*^{-/-} mice (n = 3) mice.
- (B) Representative flow plots of the thymocytes by CD4 and CD8 staining (left) and quantification of CD4⁺CD8⁺, CD4-CD8-, CD4⁺ and CD8⁺ thymocytes in the thymus (right) from WT (n = 5) versus *PP2Cm*^{-/-} (n = 3) mice. Percentages in each quadrant were displayed.
- (C-F) Frequencies of CD8⁺ T cells (C), CD4⁺ T cells (D), B cells (E) and NK cells (F) in the spleens of WT (n = 7) versus *PP2Cm*^{-/-} mice (n = 6). Data from two independent experiments were pooled.
- (G-I) Percentages of IFN- γ ⁺ (G), TNF- α ⁺ (H) of CD4⁺ T cells and GzmB⁺ (I) of NK cells in the spleens of WT (n = 5) versus *PP2Cm*^{-/-} mice (n = 4).
- (J) Body weight of WT (n = 10) versus *PP2Cm*^{-/-} (n = 9) mice before tumor inoculation (left) and at the end of the experiments (right). Data from three independent experiments were pooled.
- (K-M) Frequencies of IFN- γ ⁺ (K), TNF- α ⁺ (L) and GzmB⁺ (M) CD8⁺ T cells in the spleens of WT (n = 7) versus *PP2Cm*^{-/-} (n = 6) mice after bearing LLC tumors. Data from two independent experiments were pooled.
- (N) Frequencies of CD8⁺ T cells in the spleens of LLC tumor mouse model after anti-CD8 antibody treatment in WT (n = 4), *PP2Cm*^{-/-} (n = 3), WT+anti-CD8 (n = 5) and *PP2Cm*^{-/-}+anti-CD8 (n = 3) mice.
- (O) Frequencies of CD8⁺ T cells in the spleens of B16-F10 tumor mouse model after anti-CD8 antibody treatment in WT (n = 5), *PP2Cm*^{-/-} (n = 4), WT+anti-CD8 (n = 4) and *PP2Cm*^{-/-}+anti-CD8 (n = 3) mice.
- (P-S) Percentages of IFN- γ ⁺ (P), TNF- α ⁺ (Q), CD107A⁺ (R) and GzmB⁺ (S) CD8⁺ T cells in *PP2Cm*^{-/-} mice fed with normal diet (n = 6) and with BCAA-low diet (n = 5).
- (T) ECAR influxes of CD8⁺ T cells from WT (n = 3) versus *PP2Cm*^{-/-} mice (n = 3) activated in culture media containing normal BCAA and no BCAA.
- (U) OCR influxes of CD8⁺ T cells from WT (n = 3) versus *PP2Cm*^{-/-} mice (n = 3) activated in culture media containing normal BCAA and no BCAA.
- (V-Z) Percentages of IFN- γ ⁺ (V), TNF- α ⁺ (W), CD107A⁺ (X), GzmB⁺ (Y) and Glut1⁺ (Z) CD8⁺ T cells from WT (n = 5) versus *PP2Cm*^{-/-} mice (n = 5) upon anti-CD3/CD28 stimulation in culture media containing normal BCAA and no BCAA with.
- Data are presented as mean \pm SEM. ns: non-significant, *p < 0.05, **p < 0.01, ***p < 0.001 by the Student's t test (A-Z). Unless indicated, data were the representative of two independent experiments.



Supplementary Figure 3. Related to Figure 2 and 3 — CD8⁺ T cells from *PP2Cm*^{-/-} mice exhibit less exhausted phenotypes with altered metabolic features.

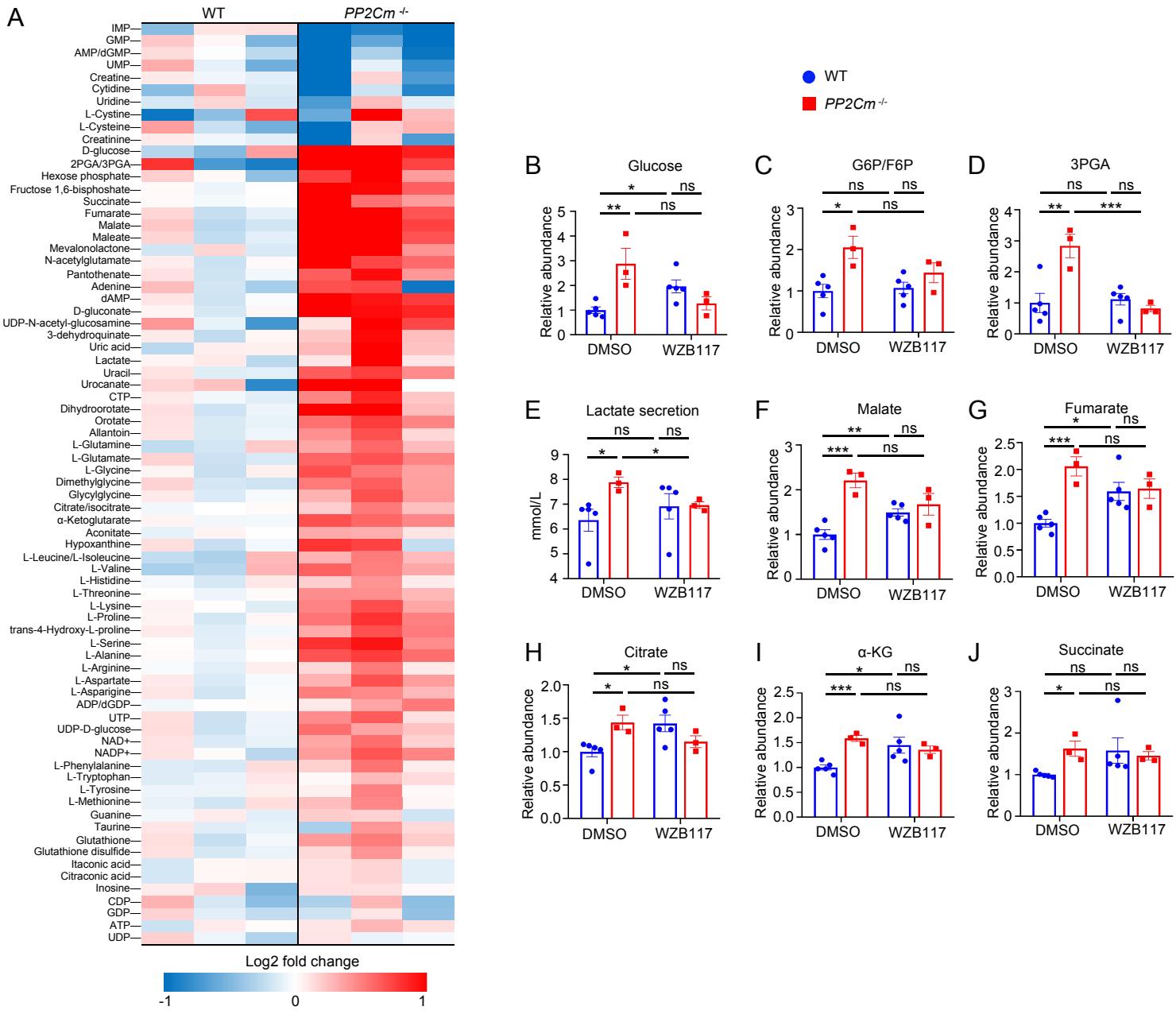
(A-D) Percentages of TCF-7⁺ (A), PD-1⁺ (B), Tim-3⁺ (C) and TOX⁺ (D) CD8⁺ T cells infiltrated in LLC tumors from WT ($n = 5$) versus *PP2Cm*^{-/-} ($n = 3$) mice.

(E-H) Percentages of IFN- γ ⁺ (E), TNF- α ⁺ (F), CD107A⁺ (G), GzmB⁺ (H) CD8⁺ T cells from WT ($n = 6$) versus *PP2Cm*^{-/-} ($n = 6$) mice upon treatment with Rapamycin versus DMSO.

(I-K) GSEA analysis indicating gene sets involved in glycolysis (I), OXPHOS (J) and fat acid oxidation (FAO) metabolism (K) in CD8⁺ T cells from *PP2Cm*^{-/-} versus WT mice.

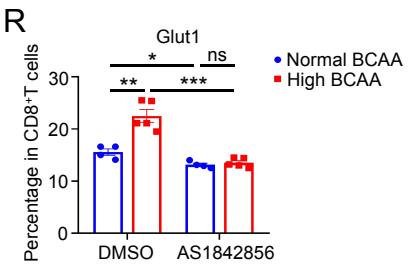
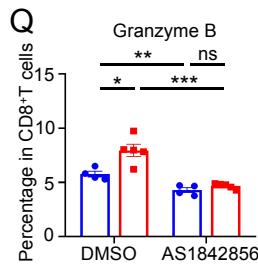
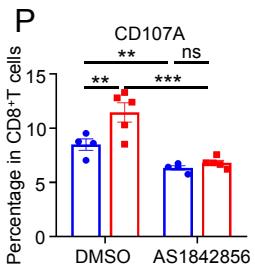
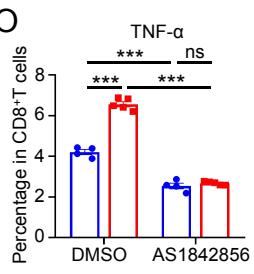
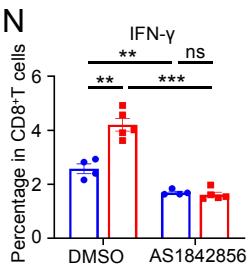
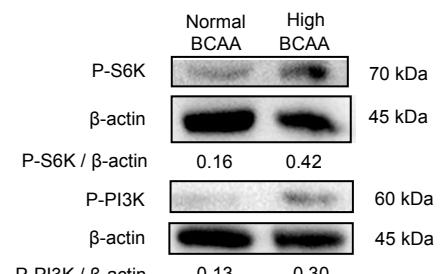
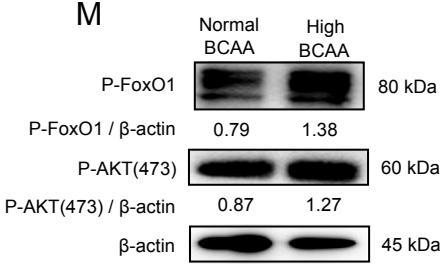
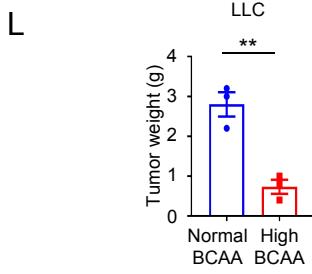
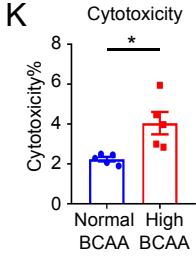
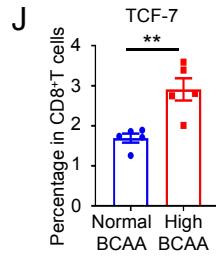
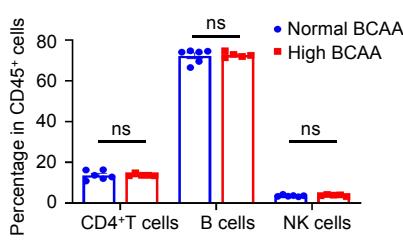
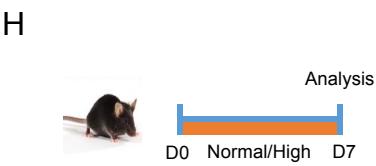
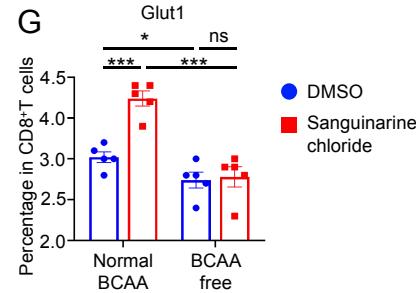
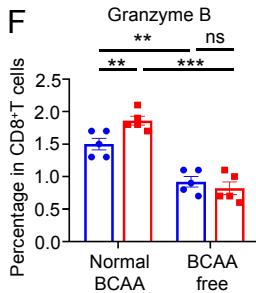
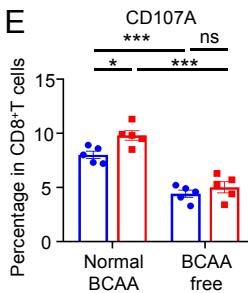
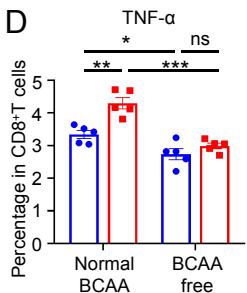
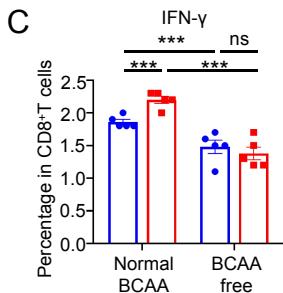
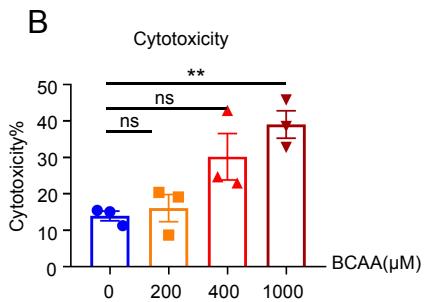
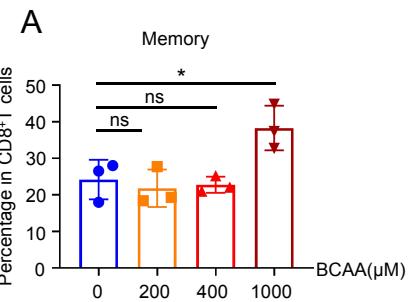
(L-P) Comparisons of *CPT1A* (L), *ACAA2* (M), *ACSS1* (N), *ACSS2* (O), *ECHS1* (P) expressions related to FAO in CD8⁺ T cells from WT ($n = 5$) versus *PP2Cm*^{-/-} ($n = 3$) mice by qPCR.

Data are presented as mean \pm SEM. ns: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by the Student's *t* test (A-H, L-P). Data were representative of two independent experiments.



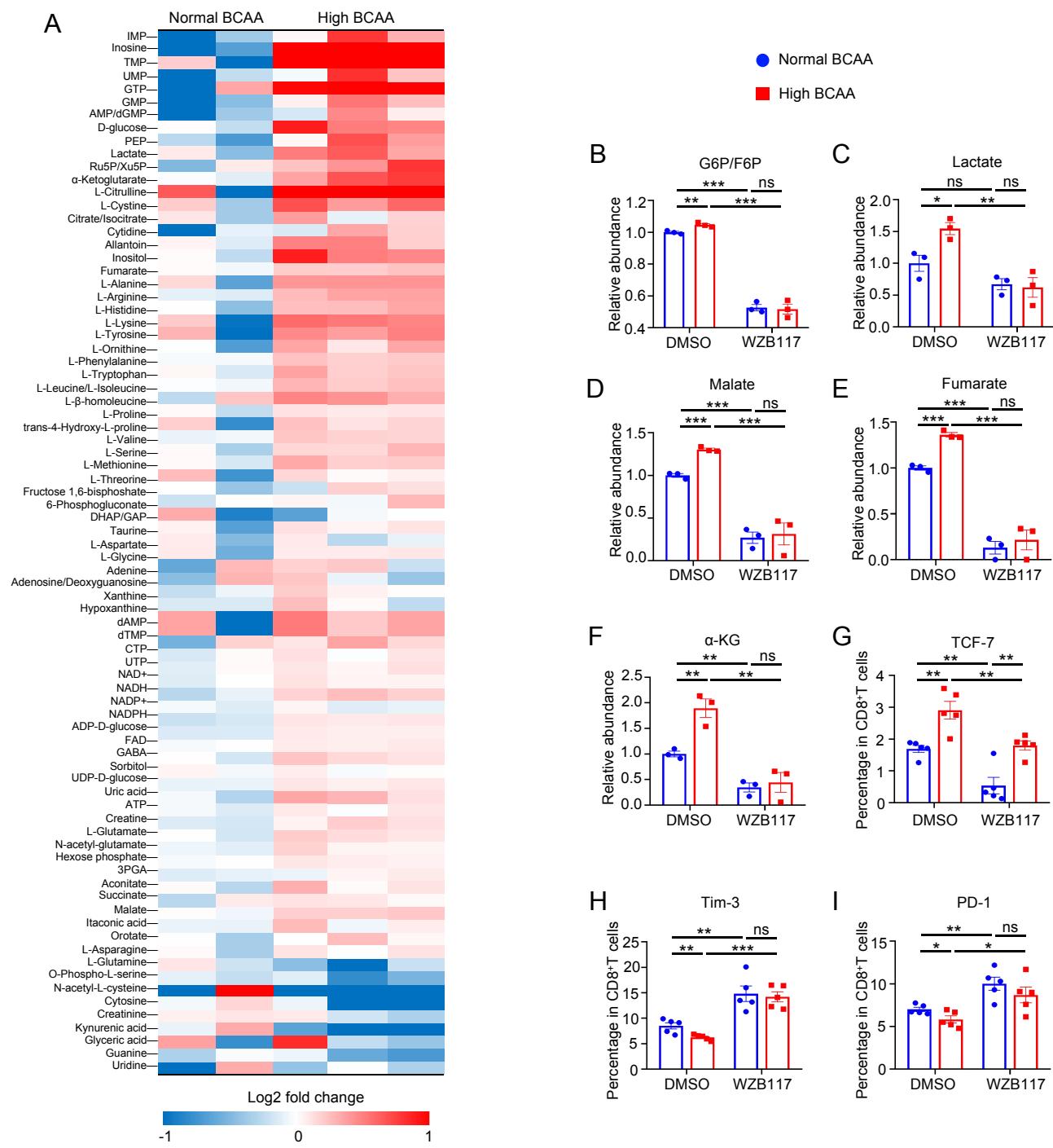
Supplementary Figure 4. Related to Figure 3 — $PP2Cm$ deficiency in CD8⁺ T cells promotes glycolysis and OXPHOS activities in a Glut1 dependent manner.

- (A) Heatmaps of intracellular metabolites in WT ($n = 3$) versus $PP2Cm^{-/-}$ ($n = 3$) CD8⁺ T cells.
- (B-J) Intermediate metabolites related to glucose consumption in anti-CD3/CD28 activated CD8⁺ T cells from WT ($n = 5$) versus $PP2Cm^{-/-}$ ($n = 3$) mice upon treatment with DMSO or WZB117.
- Data are presented as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by the Student's *t* test (B-J).
- Data were representative of two independent experiments.



Supplementary Figure 5. Related to Figure 4 — BCAA supplementation enhances CD8⁺ T cells functionality with hyper-activation of mTOR-PI3K-AKT-FoxO1 signaling axis.

- (A) Percentages of WT memory CD8⁺ T cells upon anti-CD3/28 stimulation for 24 h with BCAs at gradient concentrations. (n = 3)
- (B) Killing activities of CD8⁺ T cells to LLC cells after anti-CD3/28 stimulation for 24 h with BCAs at gradient concentrations by LDH release assay. (n = 3)
- (C-G) Percentages of IFN- γ ⁺ (C), TNF- α ⁺ (D), CD107A⁺ (E), GzmB⁺ (F) and Glut1⁺ (G) CD8⁺ T cells from WT mice (n = 5) upon anti-CD3/CD28 stimulation in culture media containing normal and no BCAs with or without PP2Cm inhibitor Sanguinarine chloride.
- (H) Experimental scheme.
- (I) Frequencies of CD4⁺ T cells, B cells and NK cells in the spleens of normal (n = 5) versus high BCAs (n = 5) fed mice.
- (J) Frequencies of TCF-7⁺ CD8⁺T cells in the spleens of normal (n = 5) versus high BCAs (n = 5) fed mice.
- (K) Killing activities of CD8⁺ T cells upon anti-CD3/28 stimulation of normal (n = 5) versus high BCAs (n = 5) fed mice to LLC cells by LDH release assay.
- (L) LLC tumor growth in *Rag*^{-/-} mice adoptively transferred with CD8⁺ T cells from normal (n = 3) and high (n = 3) BCAA-fed mice.
- (M) Immunoblotting analysis of the indicated proteins in CD8⁺ T cells from normal versus high BCAs fed mice upon anti-CD3/28 stimulation for 30 min.
- (N-R) Percentages of IFN- γ ⁺ (N), TNF- α ⁺ (O), CD107A⁺ (P), GzmB⁺ (Q) and Glut1⁺(R) CD8⁺ T cells from normal (n = 4) versus high BCAs (n = 5) fed mice after anti-CD3/28 stimulation with DMSO or AS1842856 for 72 h.
- Data are presented as mean \pm SEM. ns: non-significant, *p < 0.05, **p < 0.01, ***p < 0.001 by the *Student's t* test (A-R). Data were representative of two independent experiments.



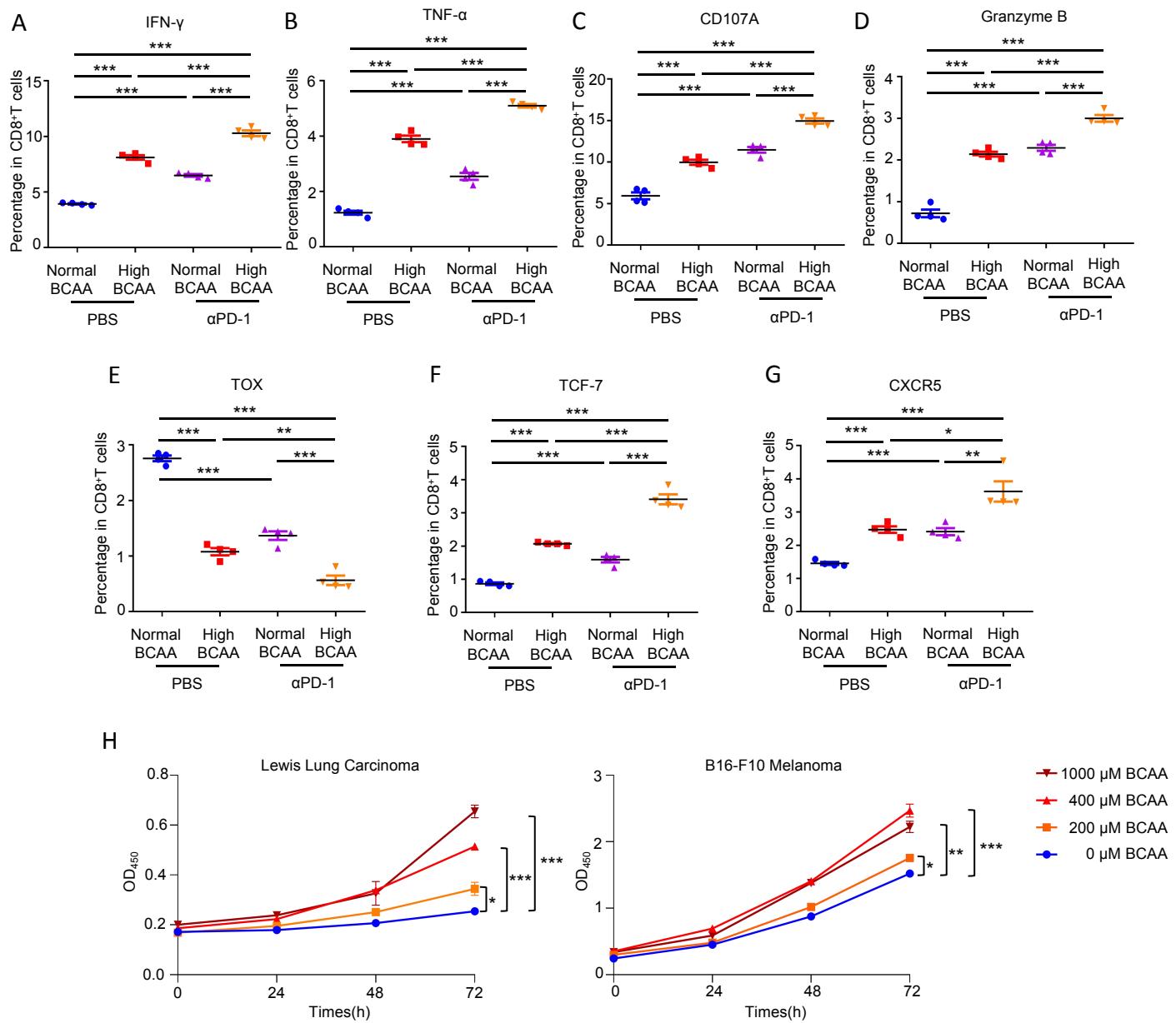
Supplementary Figure 6. Related to Figure 4 — BCAA supplementation in CD8⁺ T cells promotes glycolysis and OXPHOS activities in a Glut1 dependent manner.

(A) Heatmaps of intracellular metabolites in CD8⁺ T cells from normal (n = 2) versus high BCAs (n = 3) fed mice.

(B-F) Intracellular metabolites related to glycolysis including G6P/F6P (B), lactate (C), and the TCA-cycle activity including malate (D), fumarate (E) and α-KG (F) in CD8⁺ T cells from normal (n = 3) versus high BCAs (n = 3) fed mice upon anti-CD3/28 stimulation with DMSO or WZB117 treatment for 24 h.

(G-I) Percentages of TCF-7⁺ (G), Tim-3⁺ (H) and PD-1⁺ (I) CD8⁺ T cells from normal (n = 4) versus high BCAA (n = 5) fed mice upon anti-CD3/28 stimulation with DMSO or WZB117 for 72 h.

Data are presented as mean ± SEM. ns: non-significant, *p < 0.05, **p < 0.01, ***p < 0.001 by the Student's t test (B-I). Data were representative of two independent experiments.



Supplementary Figure 7. Related to Figure 5 — BCAA supplementation synergizes with anti-PD-1 treatment to promote anti-tumor immunity.

(A-G) Percentages of IFN- γ ⁺ (A), TNF- α ⁺ (B), CD107A⁺ (C), GzmB⁺ (D), TOX⁺ (E), TCF-7⁺ (F) and CXCR5⁺ (G) CD8⁺ T cells from normal ($n = 4$) versus high BCAs ($n = 4$) fed mice with or without anti-PD-1 treatment in B16-F10 tumor-bearing mice.

(H) *In vitro* proliferation curves of LLC (left) and B16-F10 (Right) cells with gradient BCAA concentrations by CCK-8 assays.

Data are presented as mean \pm SEM. ns: not-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by the Student's *t* test (A-H). Data were representative of two independent experiments.

Supplementary Table1 — Clinical manifestations of advanced NSCLC patients receiving nivolumab treatment with different responses

Characteristics		R (n=10)	NR (n=14)	pvalue
Age		61.90 ± 3.20	58.71 ± 2.72	0.457
Tumor diameter (mm)		55.89 ± 9.52	54.01 ± 7.48	0.876
ANC (10 ⁹ /L)		4.50 ± 0.45	4.83 ± 0.48	0.637
BPC (10 ⁹ /L)		217.50 ± 16.18	258.00 ± 17.46	0.117
ALC (10 ⁹ /L)		1.43 ± 0.12	1.36 ± 0.11	0.669
LDH (U/L)		204.78 ± 19.50	262.30 ± 26.64	0.126
Gender	Male/Female	2/8	6/8	0.252
Smoke	Non-smoker/smoker	4/6	7/7	0.635
Type	Squamous Adenocarcinom aLarge-Cell- Neuroendocrine	370	2111	0.261

R: responders, NR: non-responders, ANC: absolute neutrophil Count, BPC: blood platelets count, ALC: absolute lymphocyte count, LDH: lactate dehydrogenase.