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RESEARCH

Taurine enhances the antitumor efficacy of PD-1 antibody by boosting CD8$^+$ T cell function

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Abstract

The functional state of CD8$^+$ T cells determines the therapeutic efficacy of PD-1 blockade antibodies in tumors. Amino acids are key nutrients for maintaining T cell antitumor immunity. In this study, we used samples from lung cancer patients treated with PD-1 blockade antibodies to assay the amino acids in their serum by mass spectrometry. We found that lung cancer patients with high serum taurine levels generally responded to PD-1 blockade antibody therapy, in parallel with the secretion of high levels of cytotoxic cytokines (IFN-γ and TNF-α). CD8$^+$ T cells cultured with exogenous taurine exhibited decreased apoptosis, enhanced proliferation, and increased secretion of cytotoxic cytokines. High SLC6A6 expression in CD8$^+$ T cells was positively associated with an effector T cell signature. SLC6A6 knockdown limited the function and proliferation of CD8$^+$ T cells. RNA sequencing revealed that SLC6A6 knockdown altered the calcium signaling pathway, oxidative phosphorylation, and T cell receptor signaling in CD8$^+$ T cells. Furthermore, taurine enhanced T cell proliferation and function in vitro by stimulation of PLCγ1-mediated calcium and MAPK signaling. Taurine plus immune checkpoint blockade antibody significantly attenuated tumor growth and markedly improved the function and proliferation of CD8$^+$ T cells in a mouse tumor model. Thus, our findings indicate that taurine is an important driver for improving CD8$^+$ T cell immune responses and could serve as a potential therapeutic agent for cancer patients.

Keywords Taurine · CD8$^+$ T cell · Antitumor immunity · PD-1 blockade antibody · Calcium signaling · MAPK signaling

Introduction

CD8$^+$ T cells are crucial for the control of tumors, but CD8$^+$ T cells exhibit an exhaustion signature during the development of tumors, with high expression of inhibitory receptors (e.g., PD-1, CTLA4, and Tim-3) and low secretion of cytotoxic cytokines (e.g., IFN-γ, TNF-α, and Granzyme B) [1, 2]. Immune checkpoint antibody-mediated blocking of PD-1 is an effective strategy for the treatment of cancers, as it enhances the clinical responses of CD8$^+$ T cells [3–5]. However, many patients do not respond to PD-1 blockade antibody therapy [6–8]. Although several factors have been reported to mediate resistance to PD-1 blockade antibody therapy and impair T cell immune responses, such as β-catenin activation [9], vascular endothelial growth factor-A expression [10]–], and CD73 expression in serum exosomes [11], whether metabolites affect the therapeutic efficacy of PD1 blockade antibodies by reducing T cell antitumor function is unclear. Therefore, there is a need to investigate the key metabolites in patients who are able to mount a T cell immune response following PD-1 blockade with antibodies.

Metabolite utilization is particularly crucial for T cell antitumor responses because the metabolite profile often becomes unbalanced in patients with different cancers [12, 13]. Glucose limitation as a result of consumption by tumor cells restricts the activation, immune function, and survival of T cells [14, 15]. In addition to glucose, amino acids are critical regulators of the cytotoxic response of T cells. It

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has been shown that serum methionine levels are lower in cancer patients than in healthy donors, and methionine deficiency impairs T cell function [16]. Asparagine promotes $CD8^+$ T cell activation and antitumor responses by enhancing LCK signaling [17] and glutamine metabolism increases the cytotoxic response of $CD8^+$ T cells [18]. These studies have demonstrated the regulatory roles of amino acids in T cell immune responses. Thus, a better understanding of amino acid changes during PD-1 blockade antibody therapy is paramount for improving outcomes in cancer patients.

Taurine is a sulfur-containing amino acid in animals that exists in a free form, although it is not involved in protein synthesis [19]. As early as 1990, a study showed that taurine could promote T and B cell proliferation [20]. Taurine has also been reported to attenuate T cell apoptosis mediated by activation-induced cell death [21] and to enhance T cell immune function [22, 23]. Although taurine has been shown to affect various properties of T cells, the underlying mechanisms by which taurine regulates T cell function and the correlation between taurine and PD-1 blockade antibody therapy remain unclear.

Here, we used ultra-performance liquid mass spectrometry to uncover the significantly altered amino acids in serum of lung cancer patients receiving PD-1 blockade antibody therapy. Specifically, the serum level of taurine was greatly increased in patients with lung cancer who responded to PD-1 blockade antibody therapy. Interestingly, we found that $CD8^+$ T cells in lung cancer patients expressed high levels of the taurine transporter SLC6A6, while expressing hardly any taurine synthesis-related enzymes (CSAD and CDO1), indicating that $CD8^+$ T cells mainly rely on exogenous uptake of taurine. We further found that taurine regulates the function of T cells. RNA sequencing data and in vitro experiments showed that taurine enhanced T cell proliferation and function by enhancing oxidative phosphorylation and increasing PLCγ1-mediated calcium and MAPK signaling pathways. Collectively, we established that taurine is an important driver for improving the $CD8^+$ T cell immune response and the clinical response to PD-1 blockade antibody therapy.

Materials and methods

Human samples

Samples from 21 lung adenocarcinoma (LUAD) patients treated with chemotherapy plus PD-1 blockade antibody and several healthy donors were used in this study. Depending on the patient's clinical response, the subjects were classified into a response group ($n=15$) and a no response (NR) group ($n=5$). Information on the subjects is presented in Table S1. Peripheral blood samples from the patients and healthy donors were used in the subsequent studies. The study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (2021-KY-1105-002).

Mice and tumor models

Female C57BL/6 mice (6–8 weeks old) and BALB/c mice (6–8 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All the mice were housed under specific pathogen-free conditions. B16 (a mouse malignant melanoma cell line), and 4T1 (mouse breast cancer cell line) tumor cells (2×10^5) were injected subcutaneously into the right flank of each mouse. Tumor volumes were recorded every 2 days or 3 days. PD-1 or CTLA-4 blockade antibody (Bio X Cell, USA) was used every 3 days. The animal experiments in this study were performed in accordance with Institutional Animal Care and were approved by the Ethics Committee of Zhengzhou University.

$CD8^+$ T cell isolation, transduction, and culture

Peripheral blood mononuclear cells (PBMCs) from patients and healthy donors were obtained by density gradient centrifugation. $CD8^+$ T cells were isolated from PBMCs using CD8 magnetic beads (Miltenyi Biotec, Germany). Serum-free medium for lymphocytes (Dakawe, China) containing 100 IU cytokine IL-2, 100 U/ml penicillin, and 100 μg/ml streptomycin was used to culture the isolated $CD8^+$ T cells. $CD8^+$ T cells were activated using CD3/CD28 microbeads (Miltenyi Biotec, Germany) according to the manufacturer's instructions.

For SLC6A6 knockdown experiments, SLC6A6 siRNA was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China), and SLC6A6sh lentiviral vector was constructed by Sangon Biotech Co., Ltd. (Shanghai, China). 293 T cells were used to package the SLC6A6sh virus, and the supernatants used for transduction were collected. An empty vector was used as a control. Isolated $CD8^+$ T cells were adequately activated by CD3/CD28 microbeads for 2 days and transduced with SLC6A6 siRNA or SLC6A6sh virus.

Metabolomic

Serum from the peripheral blood of patients was isolated by centrifugation at 1,500 rpm and stored at −80℃ after flash freezing in liquid nitrogen. Serum metabolites were extracted and analyzed by Biotree Biomedical Technology Co., Ltd. (Shanghai, China). Details of the metabolite extraction, identification, and quantification methods were provided by Biotree Biomedical Technology Co., Ltd.

Flow cytometry

PBMCs from patients, isolated $CD8^+$ T cells, and single-cell suspensions from spleen and tumor tissues in mice were prepared for flow cytometry.

For T cell cytotoxic cytokine staining, cells were plated in 24-well plates and stimulated with PMA (Sigma, USA) and ionomycin (Sigma) for 4 h. The cells were incubated with CD8 antibody (BioLegend, USA) for 15 min at 4 °C and fixed with 4% paraformaldehyde (Beyotime, China). After being washed twice with phosphate-buffered saline (PBS), the cells were permeabilized with 1 × permeabilization buffer (BioLegend, USA) for 30 min at room temperature. The cells were incubated with IFN-γ (BioLegend, USA) and TNF-α (BioLegend, USA) antibodies for 15 min at 4 °C, followed by two washes with PBS, and the cells were then resuspended in a final volume of 200 μL flow buffer and analyzed by flow cytometry.

For Ki67 staining, the cells were incubated with CD8 antibody (BioLegend, USA) for 15 min at 4 °C. The cells were then fixed with 4% paraformaldehyde (Beyotime, China) and permeabilized with 1 × permeabilization buffer (BioLegend, USA). After being washed twice with PBS, Ki67 antibody (BioLegend, USA) was used for cell staining at 4 °C. The cells were resuspended in a final volume of 200 μL flow buffer after 15 min and analyzed by flow cytometry.

For mitochondrial detection, collected T cells were incubated with MitoTracker™ Deep Red FM (Thermo Fisher Scientific, USA) for 30 min at 4 °C. Then, cells were washed two times using PBS. Finally, staining cells were resuspended in a final volume of 200 μL flow buffer after 15 min and analyzed by flow cytometry.

For detection of MAPK signaling activation, cells were fixed with 4% paraformaldehyde (Beyotime, China). After being washed twice with phosphate-buffered saline (PBS), the cells were permeabilized with 1 × permeabilization buffer (BioLegend, USA) for 30 min at room temperature. Then, cells were incubated with p38 (Cell Signaling Technology, USA) and p44/42 (Cell Signaling Technology, USA) antibodies for 15 min at 4 °C, followed by two washes with PBS. Fluorescent secondary antibodies were used to incubate with staining cells for 15 min at 4 °C. Finally, cells were resuspended in a final volume of 200 μL flow buffer and analyzed by flow cytometry.

Imaging cytometry

Cultured $CD8^+$ T cells were collected and washed for detection of NFAT nuclear translocation. T cells were incubated with CD8 antibody (BioLegend, USA) for 15 min at 4 °C and fixed with 4% paraformaldehyde (Beyotime, China). After being washed twice with phosphate-buffered saline (PBS), the cells were permeabilized with 1 × permeabilization buffer (BioLegend, USA) for 30 min at room temperature. Then, T cells were incubated with NFAT (Cell Signaling Technology, USA) antibody for 15 min at 4 °C, followed by two washes with PBS. Fluorescent secondary antibodies were used to incubate with staining cells for 15 min at 4 °C. After washing 2 times, cells were resuspended in a final volume of 20 μL flow buffer and added hoechst (Beyotime, China) for staining nuclear before detection. IDEAS Application v6.0 was used to analyze NFAT nuclear translocation.

Enzyme-linked immunosorbent assay (ELISA)

Diacylglycerol (DAG) in T cell was determined by ELISA according to specification. Firstly, collected cells were lysed in RIPA buffer (Beyotime, China). Cell lysate and standards were added to the assay plate. Then, horseradish peroxidase-labeled competing antigens were added and mixed with cell lysate or standards for 60 min at 37 °C. After incubation, assay plate was washed 5 times by washing buffer. Substrates A and B were added to the plate for 15 min at 37 °C, respectively. Finally, termination solution was added into assay plate for 15 min. Absorbance values at 450 nm were measured using SpectraMax iD3 (Molecular Devices, USA).

RNA isolation, transcriptome profiling, and quantitative real-time PCR

Total RNA from $CD8^+$ T cells was extracted using TRIzol® reagent (Takara, Japan) according to the manufacturer's instructions. The $CD8^+$ T cell mRNA was reverse-transcribed using HiScript® III All-in-one RT SuperMix Perfect for qPCR (Vazyme, China) and processed for quantitative real-time PCR with FastStart Essential DNA Green Master (Roche, USA). β-actin was used as the housekeeping gene. Relative gene expression (ΔΔCt) was determined by normalization to β-actin.

RNA sequencing

$CD8^+$ T cells in the NC and SLC6A6 groups were collected and stored at −80 °C. mRNA was extracted, and RNA sequencing was performed using BGI Genomics (Shenzhen, China). DESeq2 ref. [45] was used to perform differential expression analysis, and genes were considered to be significantly differentially expressed at an FDR $P < 0.05$. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Set Enrichment Analysis (GSEA) were performed at https://biosys.bgi.com.

Western blotting

Total cellular proteins were extracted from cells by lysis and ultrasonication in RIPA buffer (Beyotime, China). The cells were then centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant was transferred to an empty 1.5 mL tube. The protein concentration was measured using the bicinchoninic acid (BCA) method. Loading buffer (Beyotime, China) was added to all of the samples, which were then heated for 10 min at 100 °C. SDS–PAGE gel electrophoresis was performed at 200 V for 30 min. Transfer to 0.45 μm PVDF membranes (GE Healthcare, USA) was carried out at 400 mA for 30 min. The membranes were then blocked with 5% skimmed milk powder at room temperature for 1 h. Primary antibodies were incubated with the membranes at 4 °C overnight after washing with Tris-buffered saline with Tween 20 (TBST). The next day, the membranes were incubated at room temperature for 1 h and then washed three times with TBST. The washed membranes were then incubated with secondary antibodies at room temperature for 1 h. Finally, after being washed three times with TBST, the target proteins were detected using SuperSignal West Pico PLUS (Thermo Fisher Scientific, USA) and imaged using a FluorChem R system (ProteinSimple, USA).

Calcium flux assay

$CD8^+$ T cells were washed with PBS and loaded with Fluo-4AM (Beyotime, China) according to the manufacturer's instructions. Briefly, $CD8^+$ T cells were incubated with Fluo-4AM at 37 °C for 30 min and then washed with PBS. The washed $CD8^+$ T cells were resuspended in flow buffer, and calcium flux was determined by flow cytometry. FlowJo V7 software was used to analyze calcium flux.

Seahorse assay

Oxidation phosphorylation of SLC6A6 knockdown or taurine treatment $CD8^+$ T cells were determined using XF96 extracellular flux analyzer (Agilent Technologies, USA) according to the user guide. The sensor cartridge in XF Calibrant was hydrated at 37 °C overnight in a non-CO_2 incubator. Next day, T cells were seeded in the cell culture plate at 5×10^5 cells/well. Meanwhile, compounds used in oxygen consumption rate (OCR) were prepared and added into sensor cartridge. Finally, cell culture plate and sensor cartridge were loaded into XF96 extracellular flux analyzer for OCR detection.

Statistical analysis

The data were analyzed using GraphPad Prism 8 software. Comparisons between two groups were performed using Student's *t* test and nonparametric tests. Multiple group comparisons were performed using a one-way analysis of variance. p value < 0.05 was considered to be statistically significant. All statistical measurements are presented as means ± the standard error of the mean (SEM).

Results

Elevated taurine levels in lung cancer patients responding to PD-1 blockade antibody therapy

Many studies have reported that amino acids are critical for T cell antitumor immunity [24]. To investigate the levels of amino acids in the serum of patients, metabonomic was performed on the serum of R and NR LUAD patients treated with PD-1 blockade antibodies. Among the organic acids and derivatives comprising most amino acids, the R group patients had relatively high levels of two amino acids (taurine and methylhistidine), and taurine displayed the largest increase (Fig. 1A). TCGA data of LUAD patients were downloaded and used to analyze the effects of metabolic pathways on T cells (Table S2). Our analysis revealed that taurine and hypotaurine metabolism were positively associated with $CD8^+$ T cells (Fig. 1B). These results demonstrated that differential amino acids might be involved in regulating T cell immunity. In addition, we found that $CD8^+$ T cells in the R group produced more cytotoxic cytokines (IFN-γ and TNF-α) than those in the NR group (Fig. 1C).

Taurine enhances T cell antitumor immunity

To investigate whether taurine directly influences T cell antitumor properties, we added different concentrations of taurine to culture $CD8^+$ T cells. The normal concentration of taurine in adult blood ranges from 10 to 100 μM according to the Human Metabolome Database (HMDB). Therefore, T cells were cultured in the presence of 30, 60, and 120 μM taurine. The number of $CD8^+$ T cells increased significantly after culture with 60 μM taurine, whereas 30 and 120 μM did not affect the number of CD8 + T cells (Fig. 2A). We also investigated the influence of taurine on T cell apoptosis and the release of cytotoxic cytokines. The proportion of apoptotic T cells ($Annexin^+$ $CD8^+$ T cells) markedly declined after treatment with taurine (Fig. 2B). $CD8^+$ T cells cultured with taurine released significantly more IFN-γ and TNF-α (Fig. 2C). These results confirm that taurine enhances T cell proliferation and function.

SLC6A6 is associated with T cell activation

Taurine in the human body is either generated by self-synthesis or obtained by exogenous intake. RNA sequencing

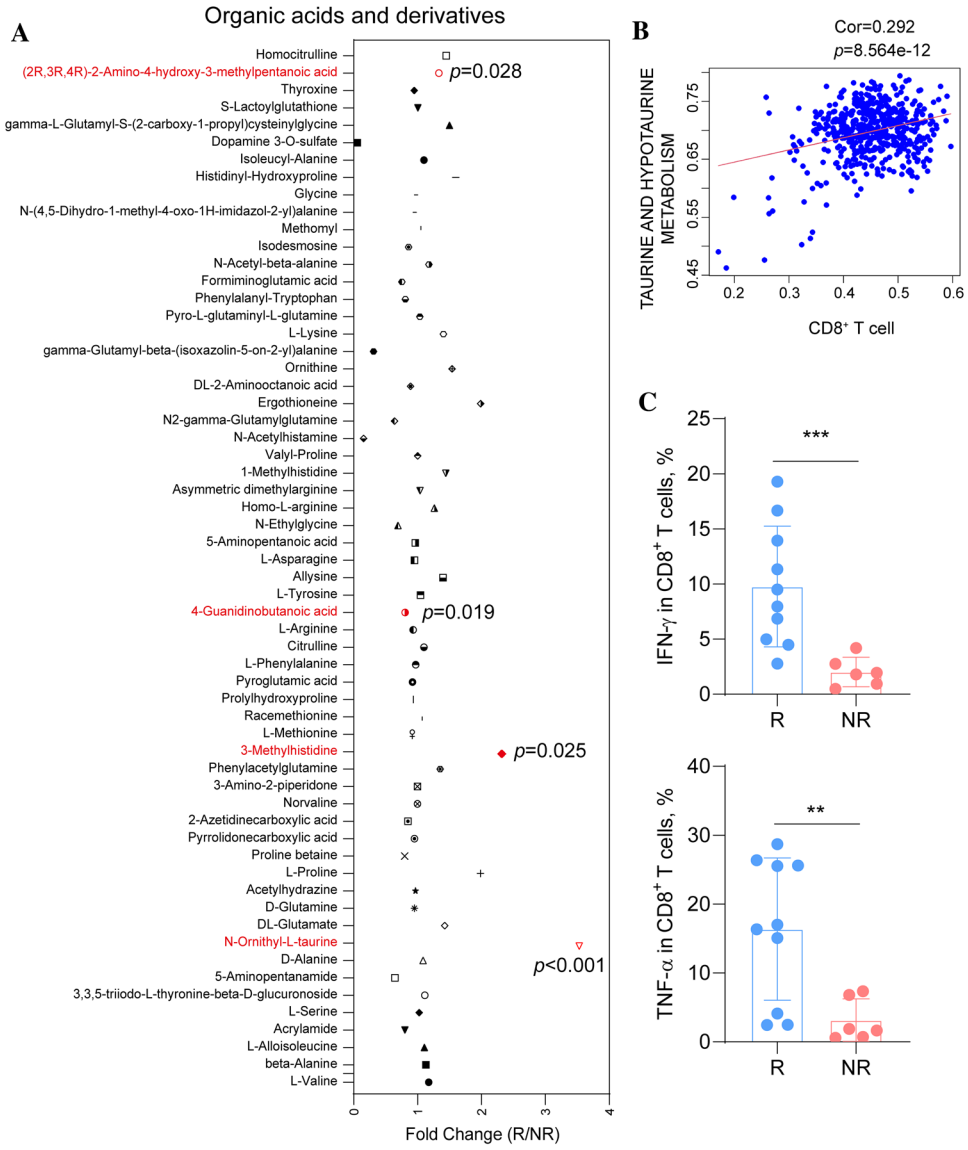

Fig. 1 Elevated taurine levels in the serum of cancer patients responding to PD-1 blockade antibody therapy. **A** Differential metabolite levels of organic acids and derivatives were analyzed in serum of lung cancer patients with PD-1 blockade antibody therapy. R, response group; NR, no response group. **B** Correlation analysis of taurine and hypotaurine metabolism and $CD8^+$ T cells in LUAD patients using TCGA. **C** Cytotoxic cytokine levels were measured in lung cancer patients with PD-1 blockade antibody therapy by flow cytometry. The data are presented as means ± SEM. Student's *t* tests and nonparametric tests were used for comparisons between two groups. $^{**}P < 0.01$, $^{***}P < 0.001$

data for T cells infiltrating into tumor tissues were downloaded from the Gene Expression Omnibus (GEO) [25]. For the analysis of taurine synthetases (CSAD and CDO1) and the taurine transporter SLC6A6, we found that T cells in lung cancer patients expressed high levels of SLC6A6 and low levels of CSAD and CDO1 (Fig. 3A), indicating that T cells in cancer patients mainly rely on the transporter SLC6A6 to obtain exogenous taurine. Using gene expression profiling interactive analysis (GEPIA), we further observed that $CD8^+$ T cells also express high levels of SLC6A6 compared to other immune cells ($CD4^+$ T cells and NK cells) in different types of cancer (Fig. 3B). In addition, by GEPIA, we found that SLC6A6 expression had a positive relationship with an effector T cell signature in different tumor types (Fig. 3C and Fig. S1). We speculate that SLC6A6 is necessary for taurine's effect on T cell antitumor functions.

SLC6A6 knockdown decreases T cell antitumor immunity

Using the http://lung.cancer-pku.cn/ website, we analyzed SLC6A6 expression in different subtypes of $CD8^+$ T cells. $CD8^+$ T cells can be divided into seven clusters based on differential gene expression. CD8-C1-LEF1 is a naive T cell cluster, and CD8-C6-LAYN is an exhaustion cluster. CD8-C2-CD28, CD8-C4-GZMK, and CD8-C5-ZNF683 are the intermediate functional states between C1 and C6. CD8-C7-SLC4A10 is a cluster with a distinct TCR[26]. The results revealed that SLC6A6 expression was upregulated upon T cell activation (Fig. 4A). Moreover, SLC6A6 expression was increased after $CD8^+$ T cell stimulation in vitro (Fig. 4B). To determine whether SLC6A6-mediated taurine uptake is critical for the T cell antitumor response, SLC6A6 expression was knocked down in activated $CD8^+$ T cells using

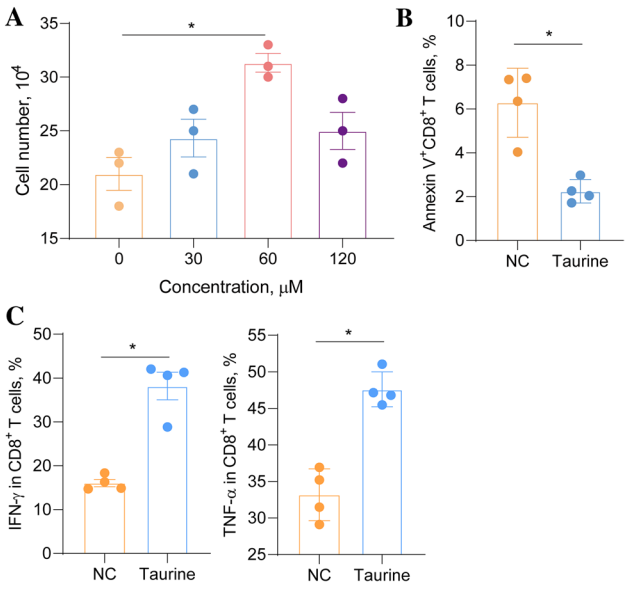

Fig. 2 Taurine promotes $CD8^+$ T cell antitumor immunity. $CD8^+$ T cells were isolated and stimulated by CD3/CD28 microbeads. **A** 1.5×10^5 activated $CD8^+$ T cells were cultured with different concentrations of taurine (0, 30, 60, and 120 μM). Cell numbers were calculated after 2 days of culture. **B, C** Apoptosis **B** and cytotoxic cytokine secretion **C** of $CD8^+$ T cells cultured in 60 μM taurine were measured by flow cytometry. The data are presented as means ± SEM. Student's *t* test was used for comparisons between two groups. $^*P<0.05$

SLC6A6 shRNA. We observed lower expression of cytotoxic cytokines (IFN-γ and TNF-α) and the cell proliferation marker Ki67 in SLC6A6sh T cells cultured in the presence of added taurine (Fig. 4C and 4D).

To confirm these results, RNA sequencing of NC and SLC6A6sh T cells was performed. Differentially expressed genes were identified and used for pathway analysis (Fig. 4E). GSEA results show that the calcium signaling pathway and oxidative phosphorylation were enriched in NC compared with SLC6A6sh (Fig. 4F). Furthermore, PD-L1 expression, the PD-1 checkpoint pathway, the T cell receptor signaling pathway, and other pathways were altered in SLC6A6sh T cells (Fig. 4G, H). PLCγ1, Akt, CaN, and other molecules associated with T cell activation were higher in NC compared with SLC6A6sh (Fig. 4H).

SLC6A6 knockdown results in decreased T cell oxidative phosphorylation

Based on GSEA analysis, OCR was measured using seahorse assay to assess oxidative phosphorylation of T cell. We found that T cell oxidative phosphorylation was decreased after SLC6A6 knockdown (Fig. 5A). Further analysis exhibited SLC6A6 knockdown-reduced basal respiration and maximal respiration of T cell (Fig. 5B). Reasonably, the addition of exogenous taurine enhanced T cell oxidative phosphorylation (Fig. 5C). Mitochondrion is the important

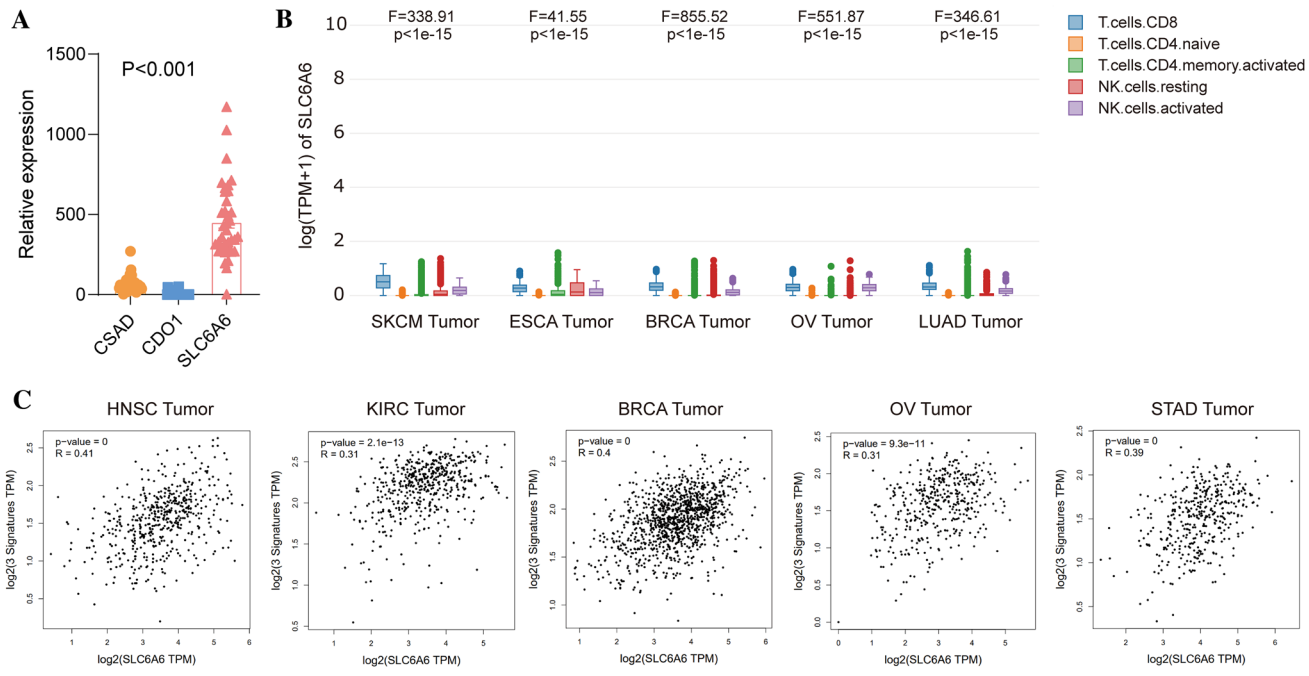

Fig. 3 SLC6A6 is highly expressed in $CD8^+$ T cells and is positively correlated with T cell activation. **A** Expression of taurine synthetases (CSAD and CDO1) and the taurine transporter SLC6A6 in lung cancer patients from GSE90730. **B** SLC6A6 expression by different immune cells was analyzed in different cancers using GEPIA. **C** The correlation between SLC6A6 and an effector T cell signature was investigated in different cancers using GEPIA. One-way ANOVA was used for multiple group comparisons

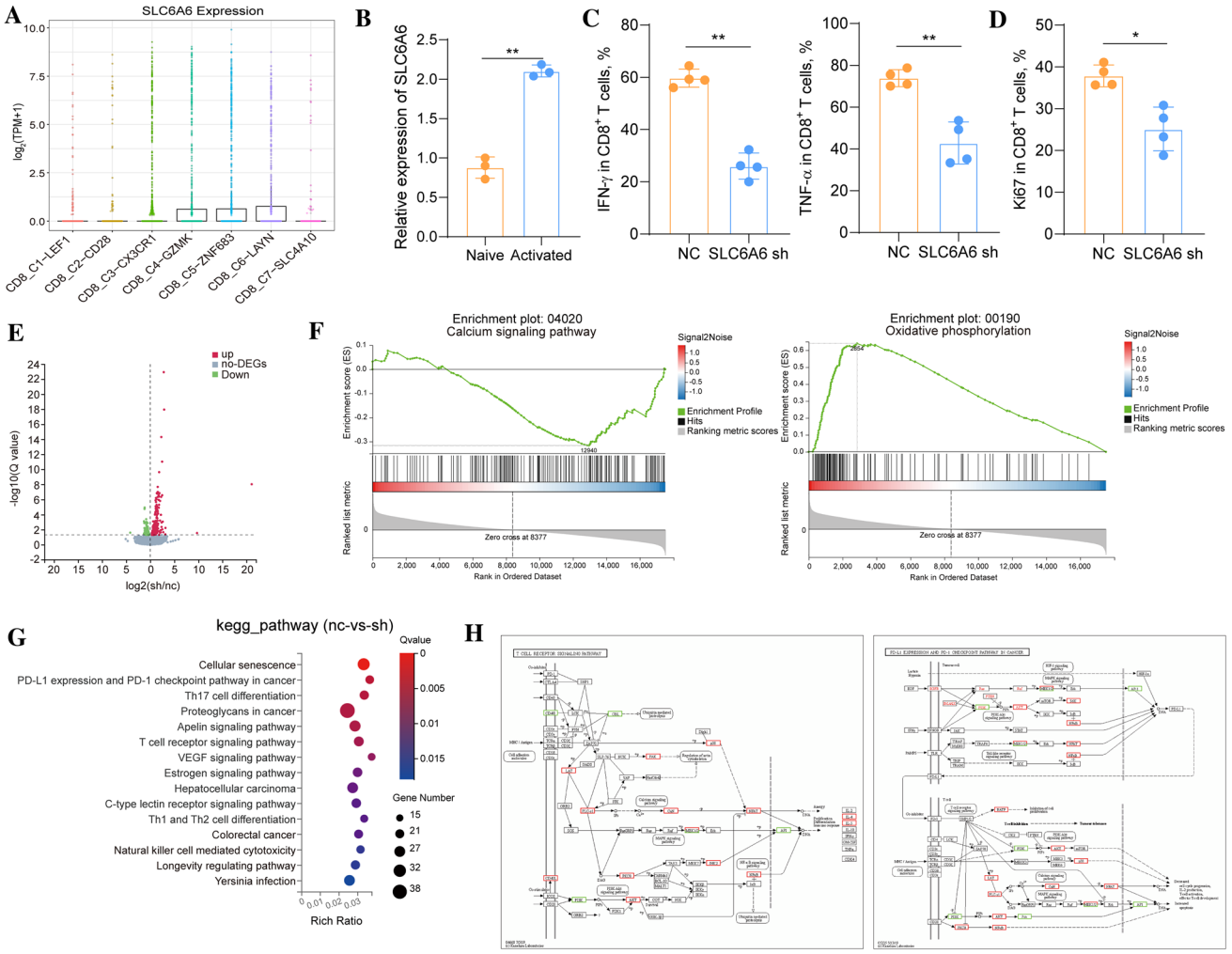

Fig. 4 SLC6A6 knockdown impairs the proliferation and antitumor function of CD8$^+$ T cells. **A** SLC6A6 expression in different subtypes of CD8$^+$ T cells based on single-cell sequencing available on http://lung.cancer-pku.cn/ website. **B** RNA of naive and activated CD8$^+$ T cells was extracted and used to assay SLC6A6 expression. **C, D** Cytotoxic cytokine secretion **C** and proliferation **D** were tested in NC and SLC6A6 knockdown CD8$^+$ T cells. RNA of NC and SCL6A6 knockdown CD8$^+$ T cells was extracted and used for RNA sequencing. **E** Differential genes were analyzed between NC and SLC6A6 sh. **F, G** GSEA **F** and KEGG **G** analysis of differential genes. **H** Differentially expressed genes in the T cell receptor signaling pathway and PD-L1 expression and PD-1 checkpoint pathway in cancer. The data are presented as means ± SEM. Student's *t* test was used for comparisons between two groups. $^*P < 0.05$, $^{**}P < 0.01$

organelle for oxidative phosphorylation. Therefore, we speculated that taurine might affect mitochondrial in T cells. Mitochondrial content in SLC6A6 knockdown T cells significantly decreased compared to NC (Fig. 5D), and exogenous taurine enhanced mitochondrial content (Fig. 5E). Overall, these results demonstrate that T cell oxidative phosphorylation and mitochondrial content were restricted in SLC6A6sh T cells.

SLC6A6 knockdown reduces PLCγ1 phosphorylation, calcium flux, and MAPK signaling

Combining GSEA and KEGG analyses, we hypothesized that the calcium signaling pathway and MAPK signaling pathway might be primary pathways influenced by taurine. Because the green fluorescent protein of the SLC6A6 shRNA plasmid interferes with the channel for calcium flow detection, SLC6A6 siRNA was used in this part of the study. As shown in Fig. 4H, we assayed PLCγ1, an upstream regulator of the calcium signaling pathway and MAPK signaling pathway, by flow cytometry. Consistent with the RNA sequencing results, PLCγ1 phosphorylation was significantly reduced in SLC6A6si T cells (Fig. 6A). The same result was observed by western blotting (Fig. 6B). We then detected the calcium signaling pathway and MAPK signaling pathway, respectively. Firstly, we found that calcium release was impaired

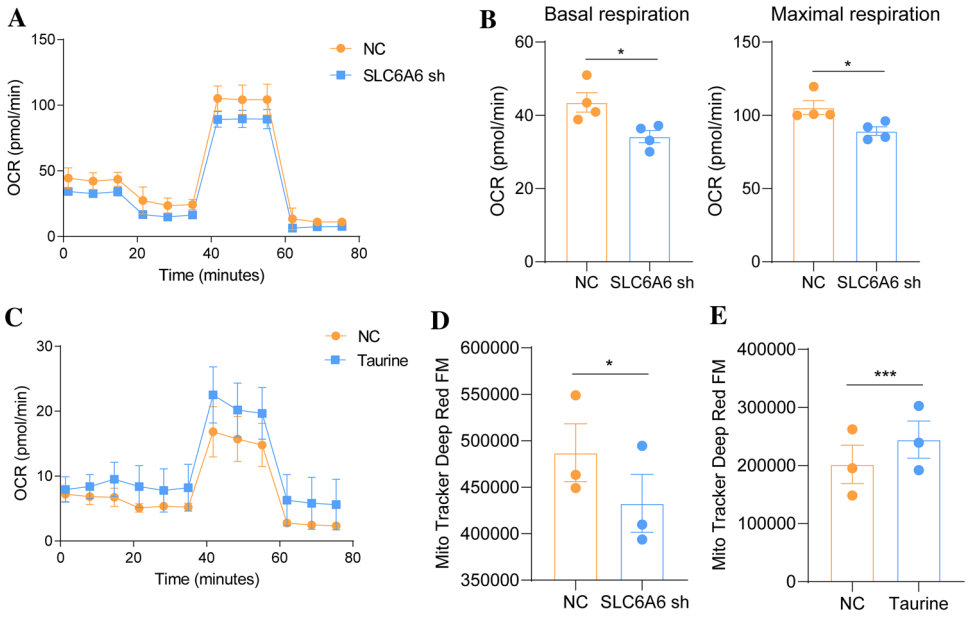

Fig. 5 SLC6A6 knockdown weakens oxidative phosphorylation of $CD8^+$ T cells. Seahorse assay was performed in SCL6A6 knockdown or taurine treated $CD8^+$ T cells to test oxidative phosphorylation. **A** Oxygen consumption rate (OCR) of SCL6A6 knockdown $CD8^+$ T cells. **B, C** Basal respiration **B** and maximal respiration **C** were detected in SCL6A6 knockdown $CD8^+$ T cells. **D** OCR in $CD8^+$ T cells was examined after adding taurine. **E, F** Mitochondrial content was detected by flow cytometry through staining Mito Tracker Deep Red FM after SLC6A6 knockdown or taurine treatment. The data are presented as means ± SEM. Student's *t* test was used for comparisons between two groups. $^*P < 0.05$, $^{***}P < 0.001$

in SLC6A6si T cells stimulated by anti-CD3 antibody (Fig. 6C), along with a decrease in the antitumor response (Fig. S2). In addition, activated $CD8^+$ T cells cultured with taurine had higher calcium concentrations compared to the NC (Fig. 6D), which further confirmed the above results. NFAT is the classical transcript factor in calcium signaling pathway. Imaging cytometry was used to detect the nuclear translocation of NFAT. Compared to NC, the percentage of NFAT nuclear translocation reduced in SLC6A6si T cells (Fig. 6E). Moreover, NFAT nuclear translocation was enhanced in T cells by adding taurine (Fig. 6F). In addition, DAG, which activates MAPK signaling pathway, is the downstream molecular of PLCγ1 in Fig. 4H. Taurine affected DAG concentration of T cells in *vitro* assay. DAG in $CD8^+$ T cells was lowered by knockdown SLC6A6 expression (Fig. 6G). And adding taurine increased DAG content in $CD8^+$ T cells (Fig. 6H). These results indicated that PLCγ-DAG axis might lead to the activation of MAPK signaling pathway. p38 and p44/42 MAPKs were detected in SLC6A6si $CD8^+$ T cells or $CD8^+$ T cells cultured with taurine. Consistent with the results of DAG, MAPK signaling pathway was inhibited by knockdown SLC6A6 (Fig. 6I) and activated by adding taurine (Fig. 6J). Taken together, these data highlight that taurine enhances the antitumor response of $CD8^+$ T cells by maintaining calcium signaling pathway and MAPK signaling pathway.

Taurine supplementation improves the therapeutic efficacy of immune checkpoint blockade antibody therapy

To validate the effect of taurine on PD-1 blockade antibody therapy, we generated tumors by implanting B16 tumor cells into the right flanks of C57BL/6 mice that were then treated with PBS, taurine, PD-1 blockade, or taurine plus PD-1 blockade. We found that tumor growth was markedly attenuated in mice treated with taurine plus PD-1 blockade, and intermediate effects were observed in mice treated with PD-1 blockade alone (Fig. 7A). Interestingly, unlike the effects on tumor growth, we observed that the release of T cell cytotoxic cytokines (IFN-γ and TNF-α) by taurine alone were greater than that by PD-1 blockade antibody therapy and was not further enhanced by taurine plus PD-1 blockade therapy in tumor site (Fig. 7B). However, the expression of the T cell proliferation marker Ki67 was markedly upregulated in mice treated with taurine plus PD-1 blockade (Fig. 7C). In addition, taurine increased T cell cytotoxic cytokines IFN-γ and TNF-α secretion, while taurine plus PD-1 blockade therapy only enhanced TNF-α secretion and did not increase IFN-γ secretion in spleen (Fig. S3A). Ki67

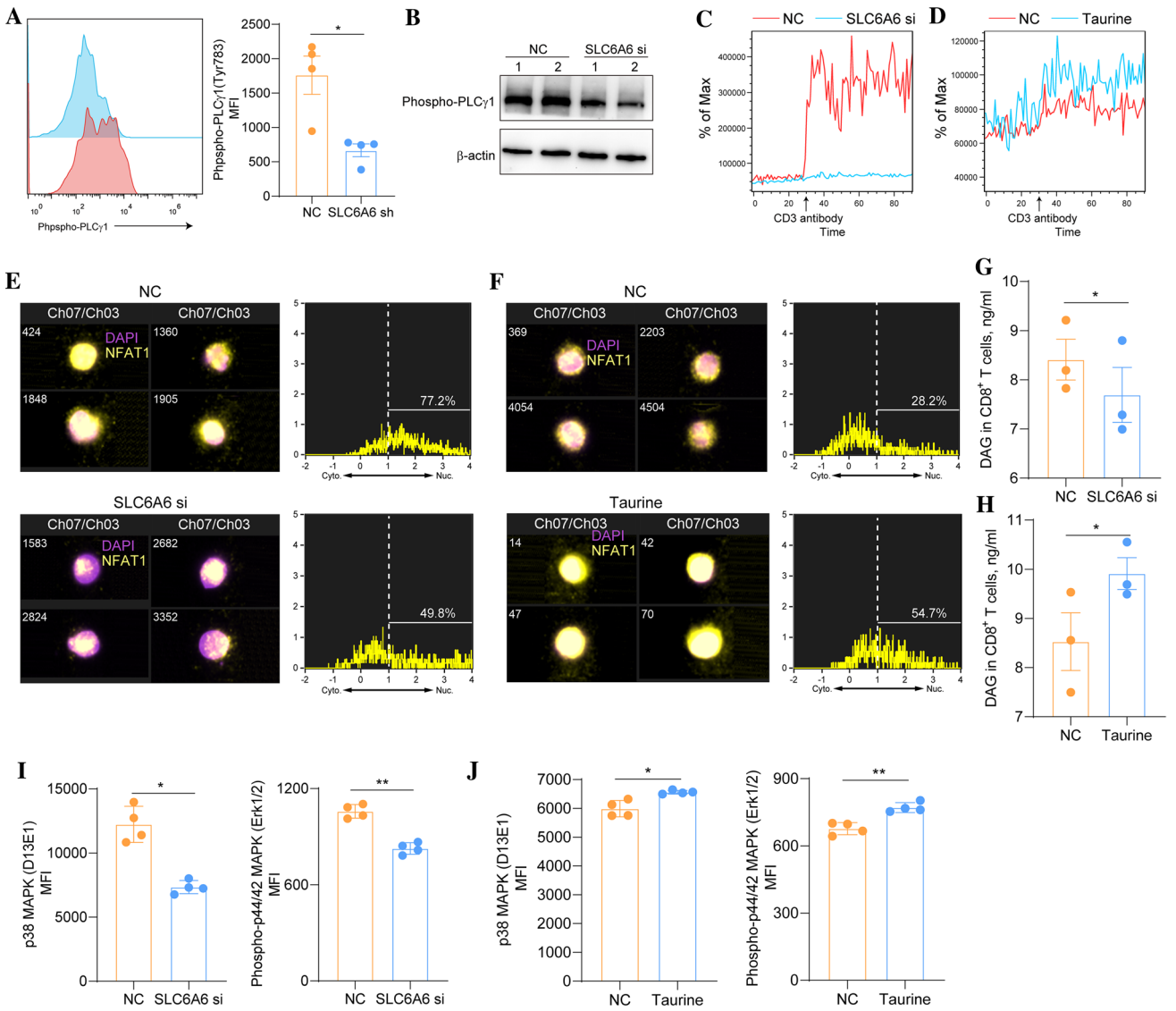

Fig. 6 PLCγ1-mediated calcium flux and MAPK signaling pathways are declined in $CD8^+$ T cells after SLC6A6 knockdown or adding taurine. **A, B** Phosphorylation of PLCγ1 in NC and SLC6A6 knockdown $CD8^+$ T cells were assayed by flow cytometry **A** and western blot **B**. **C** Intracellular calcium flux was measured in NC and SLC6A6 knockdown $CD8^+$ T cells after CD3 antibody stimulation. **D** Intracellular calcium flux was measured in NC and taurine-treated $CD8^+$ T cells after CD3 antibody stimulation. **E, F** Imaging cytometry showed NFAT nuclear translocation in SLC6A6 knockdown **E** and taurine treatment $CD8^+$ T cells **F**. **G, H** Concentration of DAG was detected by enzyme-linked immunosorbent assay (ELISA) in SLC6A6 knockdown **G** and taurine treatment $CD8^+$ T cells **H**. **I, J** Flow cytometry was used to measure p38 and phosphor-p44/42 in SLC6A6 knockdown **I** and taurine treatment $CD8^+$ T cells **J**. The data are presented as means ± SEM. Student's *t* test was used for comparisons between two groups. $^*P < 0.05$, $^{**}P < 0.01$

expressed $CD8^+$ T cell was significantly increased by taurine plus PD-1 blockade therapy (Fig. S3B). Furthermore, CD28 expression of $CD8^+$ T cell in spleen and tumor was remarkably enhanced by taurine and taurine plus PD-1 blockade therapy (Fig. S3C, D).

To further verify this phenomenon, 4T1 tumor cells were injected into BALB/c mice, and tumor-bearing mice were treated with PBS, taurine, PD-1 blockade, or taurine plus PD-1 blockade. The combination of taurine plus PD-1 blockade reduced the tumor growth (Fig. 7D). Consistent with the results in B16 tumor model, IFN-γ secretion by taurine alone was greater than that by PD-1 blockade antibody therapy and was not further enhanced by taurine plus PD-1 blockade therapy, while TNF-α secretion was significantly enhanced by taurine plus PD-1 blockade therapy (Fig. 7E). Meanwhile, taurine plus PD-1 blockade therapy also increased Ki67 expression in $CD8^+$ T cells (Fig. 7F). Due to the effect of taurine on PD-1 blockade therapy, we speculated that taurine also had the effect on other immune

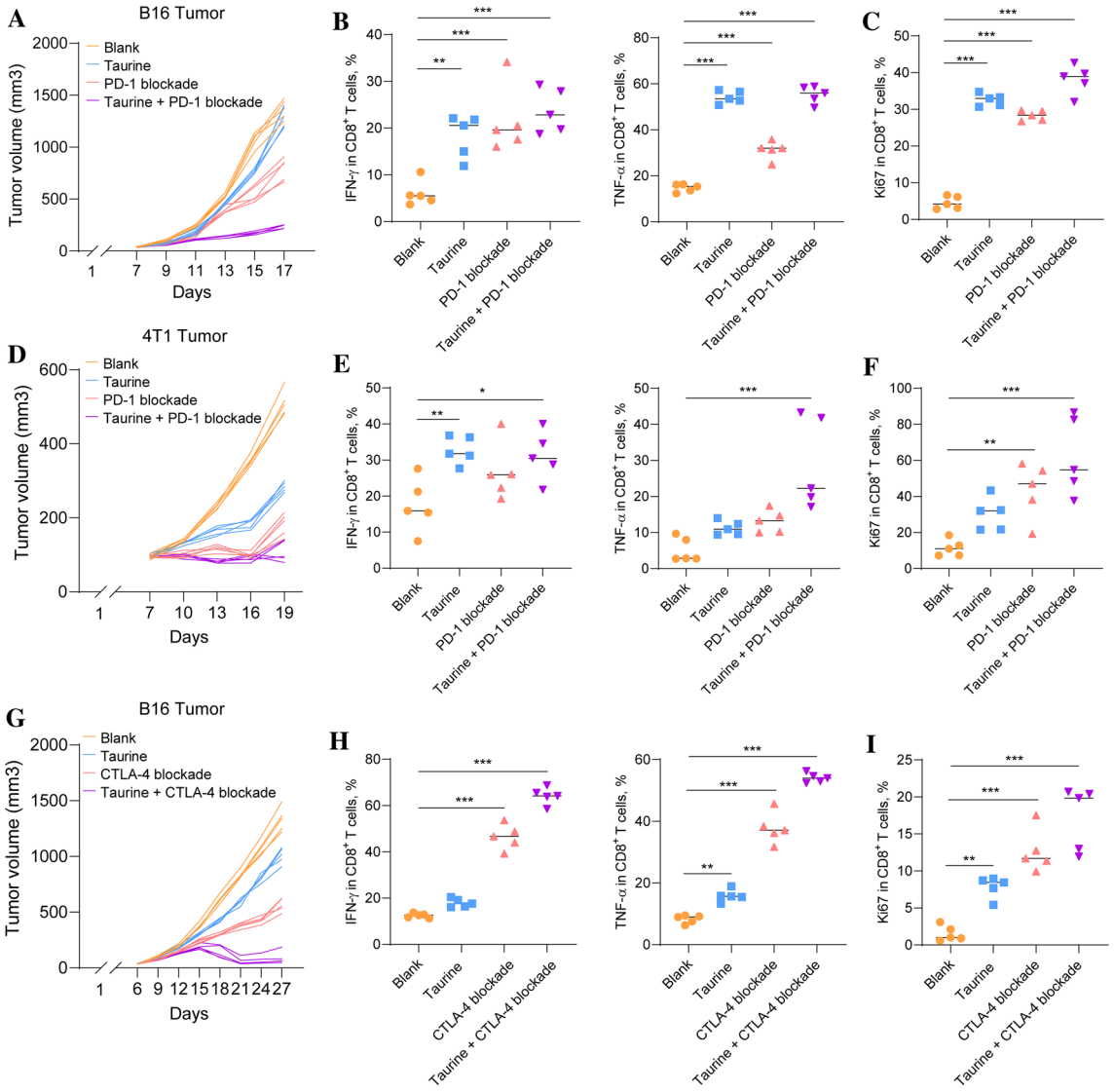

Fig. 7 Taurine plus immune checkpoint blockade antibody therapy enhances $CD8^+$ T cell antitumor immunity. **A–C** B16 tumor cells were injected into the right flanks of C57BL/6 mice treated with PBS, taurine, PD-1 blockade, or taurine plus PD-1 blockade. **(A)** Tumor growth was determined every 2 days. **B, C** Cytotoxic cytokine secretion **B** and proliferation **C** of $CD8^+$ T cells in tumor site were assayed in the four groups at 17 days. **D** Tumor growth was determined in BALB/c mice bearing 4T1 tumor. **E, F** Cytotoxic cytokine secretion **E** and proliferation **F** of $CD8^+$ T cells in tumor site were assayed by flow cytometry. **G** Tumor growth was determined in B16 tumor model treated with PBS, taurine, CTLA-4 blockade, or taurine plus CTLA-4 blockade. **H, I** Cytotoxic cytokine secretion **H** and proliferation **I** of $CD8^+$ T cells in tumor site were assayed by flow cytometry. The data are presented as means ± SEM. Student's *t* test was used for comparisons between two groups. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

checkpoint blockade therapy, such as CTLA-4. The combination therapy of taurine plus CTLA-4 blockade slowed the tumor growth in B16 tumor model (Fig. 7G). T cell cytotoxic cytokines (IFN-γ and TNF-α) section and Ki67 expression in $CD8^+$ T cells were increased by taurine plus CTLA-4 blockade therapy (Fig. 7H, I). Taken together, our results indicate that taurine supplementation can enhance the T cell antitumor response and improve the therapeutic efficacy of immune checkpoint blockade antibody therapy.

Discussion

PD-1 blockade antibody therapy represents a milestone in the field of tumor immunotherapy as it dramatically improves clinical outcomes. However, a subset of patients remains resistant to PD-1 blockade antibody therapy. Understanding cancer resistance to PD-1 blockade antibody therapy is crucial for the design of successful immunotherapies. The functional state of $CD8^+$ T cells determines the therapeutic efficacy of PD-1 blockade antibody therapy [27]. A

recent study reports that a PD-1$^+$ regulatory T cell response to PD-1 blockade antibody results in tumor progression, along with enhanced immunosuppressive factor and lower T cell antitumor function [28]. Antigen recognition disorders, abnormal T cell activation, and limited T cell infiltration are recognized as fundamental reasons for resistance to PD-1 blockade antibody therapy [29]. Upregulation of MHC-I to enhance T cell antigen recognition improves the efficacy of PD-1 blockade antibody therapy [30]. In our study, we also showed that the CD8$^+$ T cell antitumor response was impaired in NR patients compared to that in R patients, demonstrating that poor CD8$^+$ T cell function can lead to resistance to PD-1 blockade antibody therapy.

There are many factors that affect the activation and function of T cells. Amino acids are key nutrients for maintaining T cell antitumor immunity[31]. It is unclear which amino acids are altered in patients with resistance to PD-1 blockade antibody therapy. Here, we showed that the availability of amino acids could impact the T cell antitumor response. Using mass spectrometry, taurine levels were found to be significantly different between R and NR patients. A previous study has found that taurine is markedly decreased in pancreatic cancer patients compared to healthy controls [32], suggesting that taurine is involved in immunoregulation. To date, taurine has been studied in cardiovascular diseases [33], neurological disorders [34], and retinal degeneration [35]. We focused on the effects of taurine in cancer patients. It is likely that taurine controls CD8$^+$ T cell function to improve PD-1 blockade antibody therapy.

It has been reported that taurine can attenuate T cell apoptosis mediated by activation-induced cell death and enhance T cell immune function [21–23]. Consistent with these studies, our results indicate that taurine supplementation enhanced CD8$^+$ T cell proliferation and cytotoxic cytokine secretion and decreased CD8$^+$ T cell apoptosis. We also observed that the taurine transporter SLC6A6 was highly expressed in CD8$^+$ T cells, indicating that SLC6A6 mediates taurine uptake in CD8$^+$ T cells. SLC6A6 deficiency can lead to inadequate taurine uptake in retinal degeneration and cardiomyopathy [36]. SLC6A6-mediated taurine uptake also regulates macrophage polarization [37] and protects osteocytes against cell death [38]. These findings support our finding that taurine uptake is mediated by SLC6A6. Following SLC6A6 knockdown, CD8$^+$ T cells exhibited reduced secretion of cytotoxic cytokines and proliferation in the presence of taurine.

In addition, RNA sequencing results showed that the calcium signaling pathway and oxidative phosphorylation were influenced by SLCS6A6 knockdown in CD8$^+$ T cells. Through KEGG analysis, we further found that PLCγ1 was decreased by SLCS6A6 knockdown in CD8$^+$ T cells, thereby attenuating the calcium and MAPK signaling pathways. Intracellular calcium signaling is critical for triggering CD8$^+$ T cell antitumor responses [39]. PLCγ1 is an important enzyme involved in T cell signal transduction, and it has also been reported to stimulate calcium signaling [40]. In line with these findings, using flow cytometry and western blotting, we found that PLCγ1 phosphorylation and intracellular calcium flux declined in CD8$^+$ T cells after SLC6A6 knockdown. Taurine supplementation enhanced the intracellular calcium flux of CD8$^+$ T cells. p44/42 belongs to MAPK family and is reported to enhance T cell antitumor function [41]. We also found that MAPK signaling pathway was enhance by taurine, which is consistent with published research.

Owing to the limitations of PD-1 blockade antibody therapy, combination therapies have emerged as an effective way to improve therapeutic efficiency (42–45). In our model, we found that the PD-1 blockade antibody plus taurine significantly slowed tumor growth. Moreover, CD8$^+$ T cell proliferation and function in response to PD-1 blockade were boosted by taurine, highlighting the potential of exploiting taurine in PD-1 blockade antibody therapy. Our findings also help explain the diminished response of CD8$^+$ T cells in cancer patients who exhibit resistance to PD-1 blockade antibody therapy.

In summary, using mass spectrometry, our study determined that the amino acid taurine is highly expressed in R patients. We also demonstrated that taurine enhances CD8$^+$ T cell proliferation and function in vitro. We found that cancer patient CD8$^+$ T cells expressed high levels of SLC6A6 in response to taurine uptake, and the T cell antitumor response declined after SLC6A6 knockdown. RNA sequencing data and in vitro experiments showed that taurine enhanced T cell proliferation and function by enhancing oxidative phosphorylation and stimulating PLCγ1-mediated calcium and MAPK signaling pathways. Finally, taurine improved the therapeutic efficacy of immune checkpoint blockade by increasing CD8$^+$ T cell antitumor immunity in a mouse tumor model. Overall, our study provides new insights regarding immune checkpoint blockade antibody therapy resistance and suggests that taurine could serve as a potential therapeutic agent for cancer patients.

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Author contributions All authors contributed to the study conception and design. This study was designed and supervised by YZ and YP. Data collection and analysis were performed by YP, JS, FL, LW and ZL. Material preparation was performed by YL, JL, DY and LW. The first draft of the manuscript was written by YP. The manuscript was edited by XC and YZ. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets in current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Conflict of interests The authors have no relevant financial or non-financial interests to disclose.

Ethical approval This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (2021-KY-1105–002).

Informed consent Informed consent was obtained from all individual participants included in the study.

Human or animal rights All animal experiments were approved by the Animal Care and Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

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