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Glut3 overexpression improves environmental glucose uptake and antitumor efficacy of CAR-T cells in solid tumors

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ABSTRACT

Background Glucose deprivation inhibits T-cell metabolism and function. Glucose levels are low in the tumor microenvironment of solid tumors and insufficient glucose uptake limits the antitumor response of T cells. Furthermore, glucose restriction can contribute to the failure of chimeric antigen receptor T (CAR-T) cell therapy for solid tumors. However, the impact of glucose restriction remains unknown in CAR-T cell therapy.

Methods Glucose transporters were detected and overexpressed in CAR-T cells. The impacts of glucose restriction on CAR-T cells were checked in vitro and in vivo.

Results Glucose restriction significantly decreased CAR-T cell activation, effector function, and expansion. CAR-T cells expressed high levels of the glucose transporter Glut1, which has a low affinity for glucose. Overexpression of Glut1 failed to improve CAR-T cell function under glucose-restricted conditions. In contrast, the function and antitumor potential of CAR-T cells was enhanced by the overexpression of Glut3, which has the highest affinity for glucose among the Glut transporter family and is expressed in minor parts of CAR-T cells. Glut3-overexpressing CAR-T cells demonstrated increased tumoricidal efficacy in multiple xenografts and syngenetic mouse models. Furthermore, Glut3 overexpression activated the PI3K/Akt pathway and increased OXPHOS and mitochondrial fitness.

Conclusions We provide a direct and effective approach to enhance low glucose uptake levels by CAR-T cells and improve their antitumor efficacy against solid tumors.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Deprivation of glucose limits the anti-tumor functions of T cells.

WHAT THIS STUDY ADDS

⇒ Enhancing glucose metabolism increases tumoricidal potentials of chimeric antigen receptor T (CAR-T) cells.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Metabolic improvement is effective for augmenting CAR-T cell therapy.

INTRODUCTION

Chimeric antigen receptor (CAR) T cells have achieved great success in treating B-cell leukemia and lymphoma.[1] However, CAR-T cells fail to provide guaranteed benefits in treating patients with solid tumors.[1] The microenvironment of solid tumors presents many challenges to overcome for efficient CAR-T cell therapy.[2] Beyond immune suppression, metabolic suppression has emerged as an important obstacle for adoptive transfer therapy.[3] As the primary energy source, glucose and its metabolism are essential for T cells to maintain normal functions.[4 5] When stimulated, T cells increase glucose uptake and metabolism to meet the energy and biosynthesis demands for their activation, effector functions, and rapid expansion.[4 6] If glucose metabolism is inhibited, T cells demonstrate decreased effector functions and lower viability.[7] As the first step, glucose uptake determines the status of glucose metabolism in T cells. When glucose uptake decreases, T-cell function and viability sharply decrease.[8 9] In solid tumors, T cells compete with malignant and myeloid cells for glucose utility. Moreover, T cells fail to capture sufficient glucose and thus cannot fully exert their antitumor activity.[8 10 11] Thus, glucose restriction within the tumor microenvironment is a huge challenge to overcome to achieve successful T-cell-based therapy. The antitumor functions of CAR-T cells can be enhanced through various approaches, including costimulation optimization, CRISPR/cas9-mediated gene editing, cytokine overexpression, improvement of the cell culture system, and combination treatment with checkpoint blockade antibodies.[12–14] However, CAR-T cells rely on the native

machinery of T cells for activation, function, and proliferation.[15 16] They inevitably require increased glucose metabolism for optimal activation and effector functions. Indeed, enhanced glycolytic degradation of glucose is related to the augmented activation and effector functions of CAR-T cells.[12 17] Therefore, CAR-T cells require higher amounts of glucose to support increased glucose metabolism after entering tumor tissues and encountering malignant cells. It is reasonable to speculate that CAR-T cells cannot fully release their antitumor functions owing to glucose supply restriction within solid tumors, although the impact of glucose restriction remains unknown in CAR-T cell therapy.

The intake of glucose from the surrounding environment by cells is mainly dependent on glucose transporters (Gluts), which are multipass membrane proteins.[6] T cells mainly express Glut1,[18 19] with an affinity of 3–7 mM for glucose.[20] In the solid tumor microenvironment, the extracellular glucose concentrations are 2 mM or lower.[21 22] Therefore, Glut1 is unable to efficiently transport low concentrations of extracellular glucose into T cells, ultimately leading to impairment of glucose metabolism and T cells dysfunction. As modified T cells, CAR-T cells probably express Glut1 predominantly to uptake environmental glucose. Poor uptake of low glucose concentrations can also inhibit CAR-T cells. Tumor cells exploit glucose to survive and enhance immunosuppression.[23] The direct supplementation in tumors can accelerate disease progression owing to glucose consumption by malignant cells. A better approach is to improve the capacity of CAR-T cells to absorb low concentrations of glucose.

Here, we aimed to explore whether glucose restriction affected CAR-T cells. We also established a strategy to improve uptake in low glucose concentrations, thus enhancing CAR-T cell function in solid tumors. To our knowledge, this is the first modification capable of increasing glucose uptake and metabolic fitness in a direct and efficient manner to improve the antitumor function of CAR-T cells in solid tumors.

RESULTS

Glucose restriction impaired the antitumor functions of CAR-T cells

In human, the external glucose concentrations are around 5 mM in blood,[24] where CAR-T cells can efficiently eradicate hematological malignancies and result in high complete regression rates of patients. In contrast, the extracellular glucose concentrations can decrease to 2 mM or below in human solid tumors,[11 21 22] in which CAR-T cell therapy fails to produce good outcomes in the clinic. The different levels of glucose probably cause the difference in CAR-T cell therapy efficacies. To mirror the clinical conditions, 5 mM and 2 mM are chosen to determine the impact of glucose on CAR-T cells. To exclusively assess the effects of glucose availability, CD276-specific CAR-T cells with 4-1BB costimulation (CAR276-T cells) were stimulated with coated antigens in 5 or 2 mM glucose (figure 1A,B). In 2 mM glucose, CAR276-T cells expressed lower levels of activation marker CD69, and effector cytokines IFN-γ and TNF-α (figure 1C–E). Apoptosis-related markers annexin-V and active caspase three were significantly upregulated in CAR-T cells activated with 2 mM glucose (figure 1F,G). The expansion of activated CAR-T cells was altered when glucose availability was limited (figure 1H). These results indicate that glucose insufficiency could decrease the function and expansion of CAR-T cells. Next, the effect of glucose availability on the antitumor capacity of CAR-T cells was evaluated. As shown in figure 1I, CAR276-T cells were coincubated with $CD276^{+}$ Panc-1 tumor cells in 5 or 2 mM glucose. Glucose restriction had little effect on tumor cell viability (online supplemental figure S1A, B). However, glucose restriction significantly compromised the tumoricidal capacity of CAR-T cells (figure 1J). Analysis of the supernatants of the coculture system further showed that the effector function of CAR-T cells was impaired when glucose levels decreased (figure 1K). To better reflect the clinical reality, CAR276-T cells were coincubated with Panc-1 tumor cells at low effector/target (E:T) ratios for 5 days with either five or 2 mM glucose (figure 1L). CAR-T cells efficiently eradicated tumor cells in the presence of 5 mM glucose (figure 1M,N). In contrast, CAR-T cells failed to control tumor cell expansion when the glucose supply was limited (figure 1M,N), suggesting that the long-term tumor control ability of CAR-T cells is impaired when glucose is restricted. To further evaluate the effects of glucose restriction, we generated mesothelin-targeted CAR-T (CARmeso-T) cells. The CARmeso-T cells were then coincubated with mesothelin-expressing Panc-1 or H322 tumor cells in five or 2 mM glucose. Compared with 5 mM, 2 mM glucose significantly reduced the tumoricidal capacity of CARmeso-T cells (online supplemental figure S1C, D). These results imply that the antitumor functions of CAR-T cells are impaired when glucose is insufficient in the extracellular environment.

Glut3 overexpression improved glucose uptake and antitumor function of CAR-T cells

In tumor microenvironment, malignant cells and some suppressive immune cells predominantly capture glucose.[10 11] The direct supplementation may further promote the proliferation and function of tumor and immunosuppressive cells, as these cells can capture most of the glucose. Alternatively, increasing the capacity of CAR-T cells to capture low levels of glucose would specifically improve their glucose supply and antitumor function. Gluts determine the ability of cells to use glucose in the microenvironment. T cells express Glut1 and Glut3.[19 25 26] Glut1 and Glut3 have different affinities to glucose, reaching 3 mM and 1.7 mM, respectively.[20] CAR-T cells expressed high levels of Glut1, especially after activation (figure 2A). In contrast, only a small

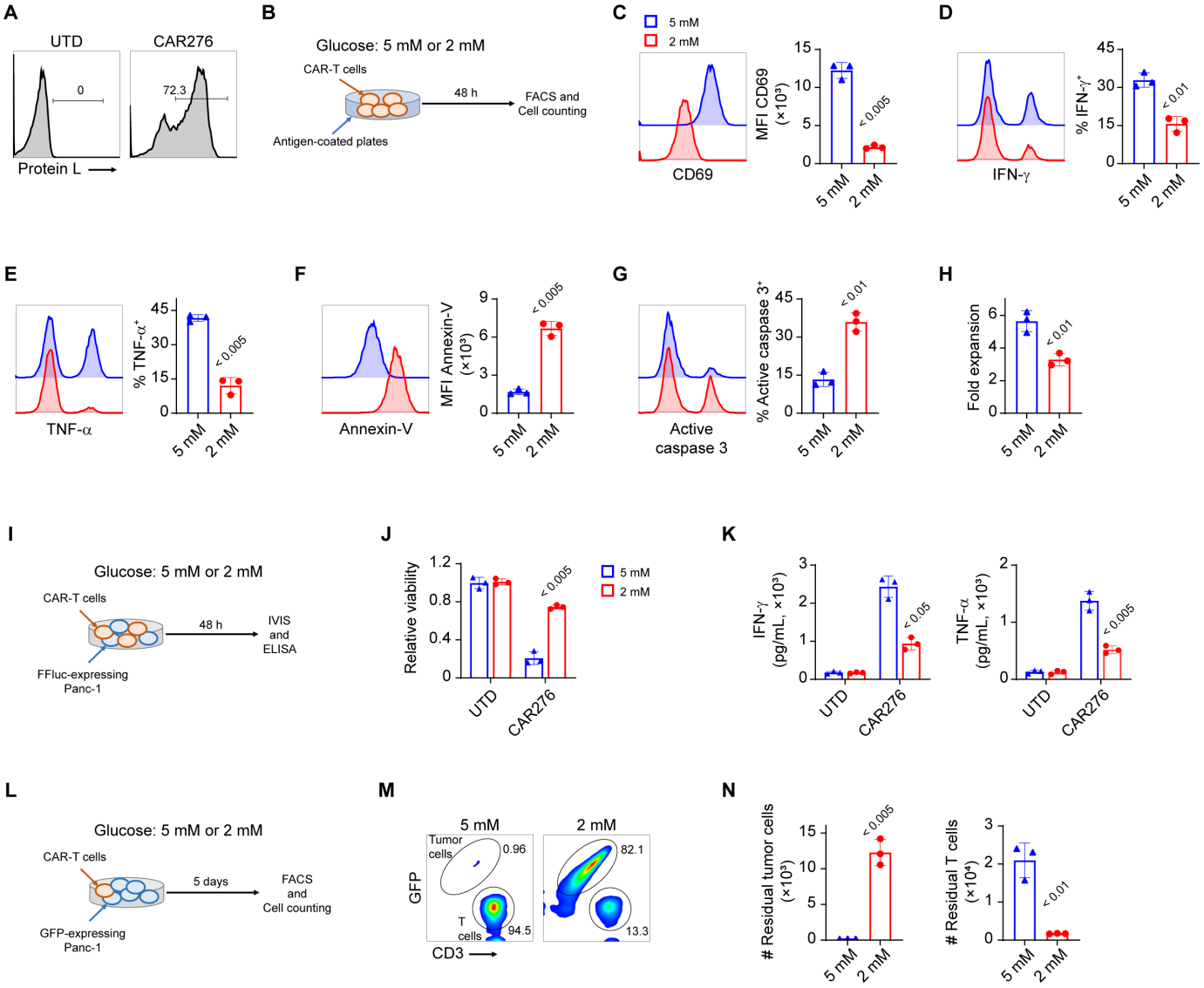

Figure 1 Glucose availability impacts activity of chimeric antigen receptor T (CAR-T) cells. (A) FACS detection of surface CAR expression by Protein L staining at 10 days post-T-cell transduction. (B–H) CAR-T cell activation, effector function, viability, expansion under different levels of glucose: CAR-T cells were activated in plates coated with human CD276 in the presence of 5 mM or 2 mM glucose (B). After 48 hours, flow-activated cell sorting (FACS) detected expression of CD69 (C), IFN-g (D), TNF-a (E), Annexin-V (F), and active caspase 3 (G). Additionally, CAR-T cells were counted and their expansions were calculated (H). (I–K) Co-culture assays, CAR-T cells were co-plated with *FFluc*+tumor cells at E:T=1:1 in media having 5 mM or 2 mM glucose for 48 hours (I). Then tumor cells bioluminescence (BLI) was detected with In Vivo Imaging System (IVIS) and then then the relative viability of tumor cells was determined (J). The supernatants were subjected to ELISA and levels of effector cytokines of CAR-T cells were determined (K). (L and N) extended co-culture assays, CAR-T cells were co-cultured with GFP$^+$ tumor cells at E:T=1:10 in media having 5 mM or 2 mM glucose for 5 days; the indicated levels of glucose were supplemented every 2 days (L). Then the residual tumor cells (GFP$^+$) and T cells (CD3$^+$) were detected by FACS (M), and their numbers were counted (N). All experiments were independently repeated three times. The representative data are shown. A two-tailed unpaired T test was used for C-H and N. Two-way ANOVA with Tukey's multiple comparison test was used for J and K. ANOVA, analysis of variance. IVIS, in vivo imaging system.

proportion of CAR-T cells expressed Glut3 (figure 2A). CAR-T cells overexpressing either Glut1 (CAR.Glut1) or Glut3 (CAR.Glut3) were generated (online supplemental figure S2A, B). Gluts overexpression did not affect CAR expression (online supplemental figure S2B). During the expansion of CAR-T cells (cultured in media with 5 mM glucose), overexpression of both Glut1 and Glut3 increased CAR-T cell proliferation and central memory polarization (CCR7$^+$CD45RO$^+$) compared with conventional CAR276-T cells (online supplemental figure S2C, D). CD4 and CD8 constituents were comparable among different CAR-T cells (online supplemental figure S2E). The glucose uptake and antitumor properties of CAR.Glut1-T and CAR.Glut3-T cells were analyzed. The Glut1 transgene specifically increased Glut1 expression (online supplemental figure S3A). Glut1 overexpression

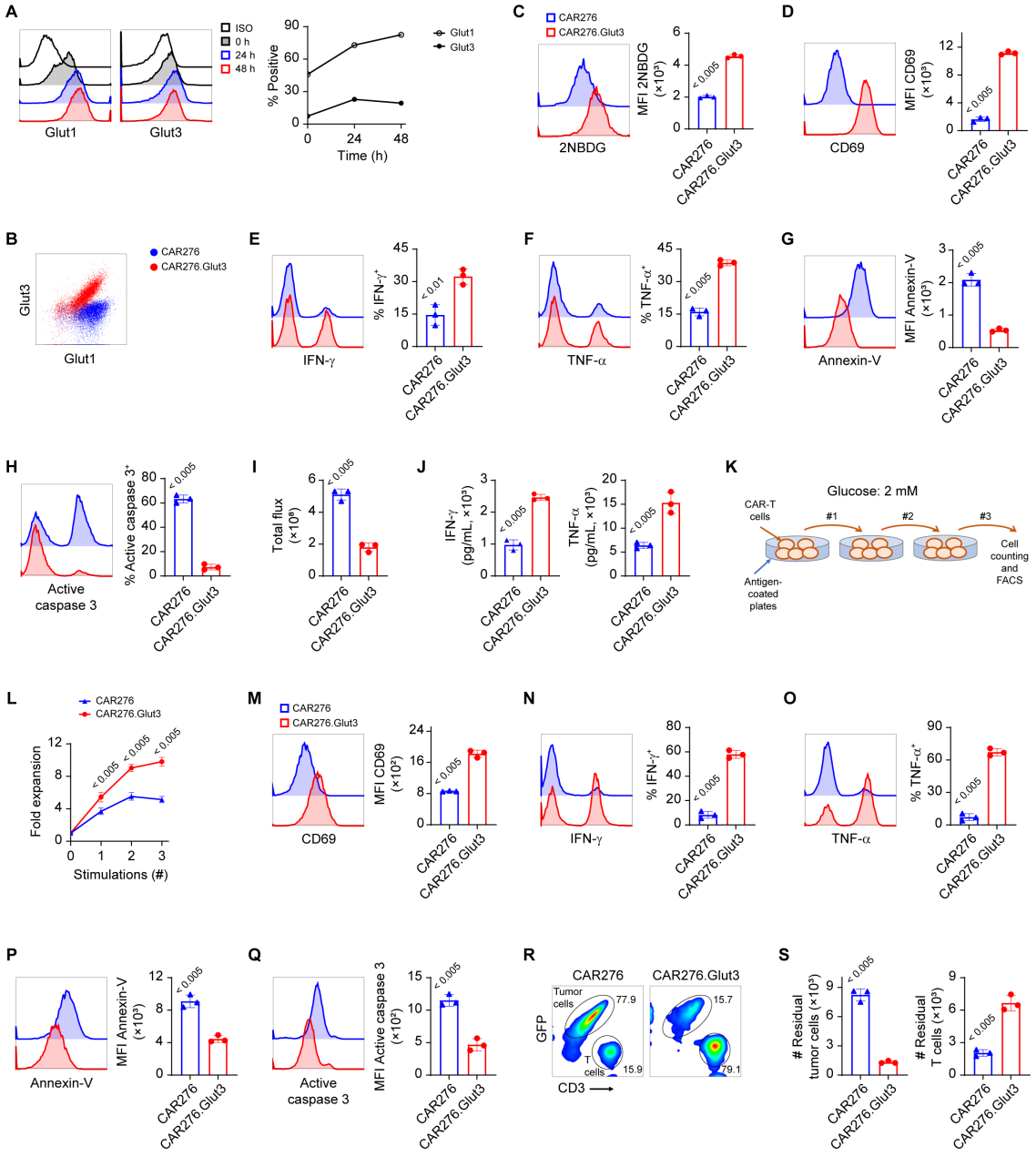

Figure 2 Glut3 overexpression improves chimeric antigen receptor T (CAR-T) cells function in vitro. (A) CAR-T cells were activated with CD3/CD28 dynabeads and subjected to flow-activated cell sorting (FACS) analysis at the indicated intervals for detection of Glut1 and Glut3. (B) CAR-T cells overexpressing Glut3 were activated for 24 hours and then subjected to FACS detection for Glut1 and Glut3. (C) CAR-T cells were activated for 24 hours, washed with glucose-free media there times, and incubated with 100 mM of 2NBDG. CAR-T cells were evaluated by FACS for 2NBDG uptake. (D–H) Activation, effector function, and apoptosis of conventional and Glut3-overexpressing CAR-T cells under glucose restriction: CAR-T cells were suspended in media with 2 mM glucose and activated in antigen-coated plates for 48 hours. Then, CD69 (D), IFN-g (E), TNF-a (F), Annexin-V (G), and active caspase 3 (H) were detected by FACS. (I–J) Tumoricidal capacity of conventional and Glut3-overexpressing CAR-T cells under glucose insufficiency: CAR-T cells were co-cultured with *FFluc*$^{+}$ Panc-1 cells at an E:T ratio of 1:1 for 48 hours in medium containing 2 mM glucose. Then, tumor cell bioluminescence (BLI) was performed (I), and effector cytokines in the supernatants were determined by ELISA (J). (K–Q) Repeated stimulation of conventional and Glut3-overexpressing CAR-T cells under glucose-limited conditions. CAR-T cells were continually stimulated in antigen-coated plates for three rounds (each round for 2 days) in media containing 2 mM glucose (K). After each round of stimulation, CAR-T cell numbers were counted and their expansion was calculated (L). After the final round of stimulation, CAR-T cells were collected and FACS was used to detect CD69 (M), IFN-g (N), TNF-a (O), Annexin-V (P), and active caspase 3 (Q). (R and S) Extended coculture assay. Conventional or Glut3-overexpressing CAR-T cells were co-culture with GFP^{+} Panc-1 cells at an E:T ratio of 1:10 for 5 days in media having 2 mM glucose. Glucose was supplemented every 2 days. At the end of the co-culture, residual tumor cells and CAR-T cells were detected by FACS (R), and their numbers were calculated (S). Representative data from three independent replicates are shown. Two-tailed unpaired T test in C–J, L, M-Q, S. Two-way ANOVA with Tukey's multiple comparison test in L. ANOVA, analysis of variance.

improved glucose analog 2NBDG uptake mildly, although the difference was statistically significant between CAR.Glut1-T and conventional CAR-T cells (online supplemental figure S3B). Consistent with the mild improvement in glucose uptake, CAR.Glut1-T cells did not show improved activation of CD69, increased effector IFN-γ and TNF-α cytokine production, or anti-apoptosis function in terms of the proportion of Annexin-V cells and active caspase 3 levels, compared with conventional CAR-T cells in 2 mM glucose (online supplemental figure S3E–I). Likely, the antitumor capacity of CAR-T cells was not improved by Glut1 in 2 mM glucose (online supplemental figure S3J, K).

Glut3 overexpressing CAR-T cells were then analyzed. CAR.Glut3-T cells showed enhanced expression of Glut3 expression and 2NBDG uptake compared with conventional CAR-T cells (figure 2B,C). Importantly, 2NBDG uptake was enhanced more efficiently in CAR.Glut3-T cells than in CAR.Glut1-T cells (online supplemental figure S3C). In consistency, the intracellular levels of glucose were significantly increased in CAR.Glut3-T cells when maintained in 2 mM glucose (online supplemental figure S3D). In 2 mM glucose, Glut3 overexpression increased CAR-T cell activation and effector cytokine expression and inhibited cell death (figure 2D–H). When coincubated with $CD276^{+}$ tumor cells, CAR.Glut3-T cells killed tumor cells more efficiently and secreted higher levels of effector cytokines (figure 2I,J). CAR.Glut3-T cells did not demonstrate tumor-killing activity when coincubated with antigen-negative tumor cells (online supplemental figure S4A). These results suggest that Glut3 overexpression does not alter recognition specificity and basal activation but improves the effector functions of CAR-T cells in a glucose-deficient environment. Additionally, CAR-T-cell expansion and function were assessed using a repeated stimulation model (figure 2K). CAR.Glut3-T cells had better expansion potential than control CAR-T cells (figure 2L). CAR.Glut3-T cells also showed better preservation of activation and effector functions after repeated stimulations (figure 2M–O). Moreover, CAR.Glut3-T cells exhibited lower levels of apoptotic death (figure 2P,Q). To further compare the persistent antitumor capacity, 5-day coincubation assays were performed in 2 mM glucose. As expected, CAR.Glut3-T cells eliminated tumor cells more efficiently than control CAR-T cells (figure 2R,S). Furthermore, CAR-T cells were checked when glucose was decreased to 0.5 mM. CAR.Glut3-T cells demonstrated enhanced activation, effector functions, and viability in comparison with the conventional CAR-T cells (online supplemental figure S4B). CD71 and CD98, markers positively indicating T cell activation and proliferation, were also increased in CAR.Glut3-T cells in 0.5 mM glucose (online supplemental figure S4B). In contrast, these properties were comparable between conventional and Glut3-overexpressing CAR-T cells when glucose was increased to 5 mM (online supplemental figure S4C). Their tumoricidal functions were also comparable when the glucose concentration was increased to 5 mM (online supplemental figure S4D), further suggesting that Glut3 improves CAR-T cells functions by enhancing uptake of low levels of external glucose. Together, these data imply that Glut3 overexpression can improve glucose uptake and antitumor functions of CAR-T cells when the glucose supply is restricted.

Glut3 overexpression altered gene transcription and activated the PI3K/Akt pathway

To comprehensively explore the impact of Glut3 overexpression, CAR-T cells were activated with 2 mM glucose and subjected to RNA sequencing (figure 3A). The transcriptional profiles of CAR.Glut3-T cells were largely different from control CAR-T cells (figure 3B). CAR.Glut3-T cells expressed higher levels of proinflammatory cytokines, including IL1A, IL-2, IL3, IL18, and TNF (figure 3C). Activation-related markers (CD44, CD69, PDCD1, and TNFRSF9) and transcription factors (BCL6, FOS, IRF8, and JUN) were also upregulated in CAR.Glut3-T cells (figure 3C). Additionally, anti-apoptotic genes transcription was elevated in CAR.Glut3-T cells (figure 3D). In contrast, control CAR-T cells showed enhanced expression of pro-apoptotic genes (figure 3D). RNA sequencing data further indicated that Glut3 overexpression could improve CAR-T cell function under glucose-restricted conditions. The PI3K/Akt pathway plays a central role in CAR-T cell activation and persistence.[27] Analysis of RNA sequencing data demonstrated that the PI3K/Akt pathway was significantly upregulated in CAR.Glut3-T cells (figure 3E). Akt, mTOR, and S6K are the key components of the PI3K/Akt pathway.[27] To confirm whether the PI3K/Akt pathway was more active in CAR.Glut3-T cells, the activation status of above-mentioned proteins was detected by flow-activated cell sorting (FACS). As shown in figure 3F–H, CAR.Glut3-T cells had higher levels of active Akt (phophos-Akt), mTOR (phophos-mTOR), and S6K (phophos-S6K) than control CAR-T cells. These results suggest that Glut3 overexpression increases PI3K/Akt pathway activity, which is critical to maintaining T cell activation and survival.

Glut3 overexpression increased global metabolism and mitochondrial function

When glucose is taken up, it is degraded through the glycolytic process to fulfill the metabolic demands of activated T cells.[4] RNA sequencing data showed that the glycolytic process pathway was enriched in Glut3-overexpressing CAR-T cells (online supplemental figure S5A). The extracellular acidification rate (ECAR) was measured to assess glucose consumption (online supplemental figure S5B). As expected, CAR.Glut3-T cells exhibited enhanced glycolysis and glycolytic capacity (online supplemental figure S5C–E). Additionally, enhanced ECAR depended on the addition and degradation of glucose, as indicated by upregulation in glycolysis phase (online supplemental figure S5C, D), further supporting the hypothesis that Glut3 enhances glucose uptake to improve CAR-T cell function. To better understand the

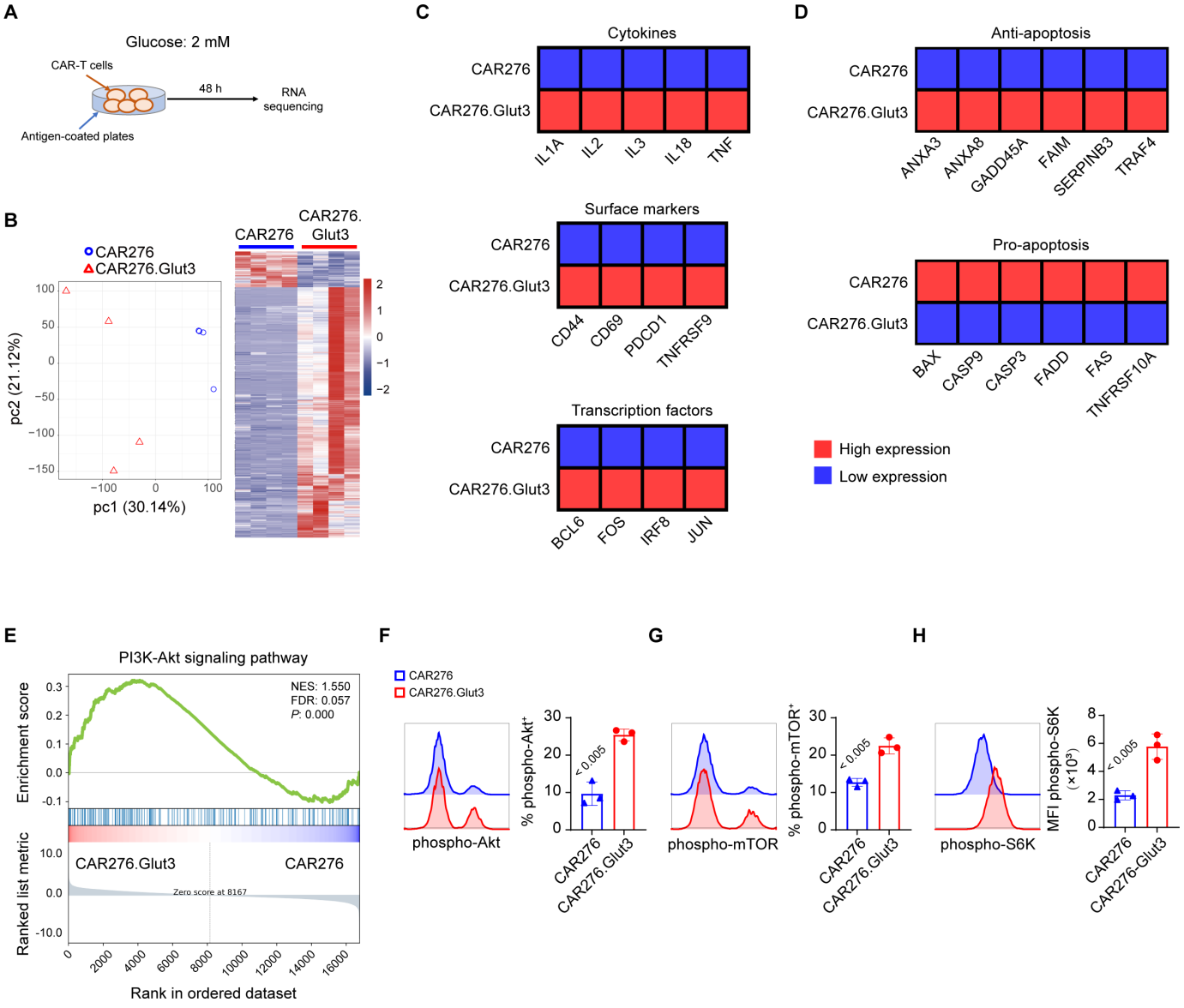

Figure 3 Glut3 overexpression alters transcriptions and elevates PI3K/Akt pathway in chimeric antigen receptor T (CAR-T) cells. (A) Schematic depiction of sample preparation for RNA sequencing. CAR-T cells were activated by plate-coated antigens for 48 hours in 2 mM glucose. Then, CAR-T cells were collected and subjected to RNA sequencing. (B) Principal component analysis and heatmap of genes transcriptions in conventional and Glut3-overexpresing CAR-T cells. (C and D) Relative expression of genes of interest in CAR276.Glut3-T cells and CAR276-T cells, according to RNA sequencing data. (E) Gene set enrichment analysis (GSEA) of the PI3K/Akt pathway in CAR-T cells. (F-H) Flow-activated cell sorting (FACS) analysis of intracellular phosphor-Akt, phosphor-mTor, and phosphor-S6K in CAR-T cells activated in antigen-coated plates for 48 hours with 2 mM glucose. CAR-T cells from four donors were subjected to RNA sequencing, and the sequencing data were pooled. In F and G, the data shown are representative of three independent experiments: two-tailed unpaired t-test.

impact of Glut3 overexpression, metabolomic assays were performed (figure 4A). Glut3 sharply changed the metabolic profiles of CAR-T cells (online supplemental figure S6A). As shown in figure 4B, 3464 metabolites were upregulated in CAR.Glut3-T cells, whereas fewer metabolites were downregulated. Differentially expressed metabolites were analyzed. As shown in figure 4C, CAR.Glut3-T cells showed a global enhancement of metabolic processes involving amino acids, lipids, nucleotides, and glucose (as indicated by central carbon metabolism in the cancer pathway). Analysis of RNA sequencing data also showed that central carbon metabolism in the cancer pathway was upregulated in CAR.Glut3-T cells (online supplemental figure S6B). In this pathway, glucose is degraded to pyruvate via glycolysis and then enters the tricarboxylic acid (TCA) cycle in the mitochondria (figure 4D). Our metabolomic data showed that the intermediate metabolite pyruvate increased in CAR.Glut3-T cells (figure 4E), which is consistent with the enhanced ECAR observed in CAR.Glut3-T cells (online supplemental figure S5C, D). Furthermore, the metabolite levels derived from the TCA cycle increased in the CAR.Glut3-T cells (figure 4E). These data suggest that Glut3 overexpression increases the TCA cycle and glucose utilization by this cycle.

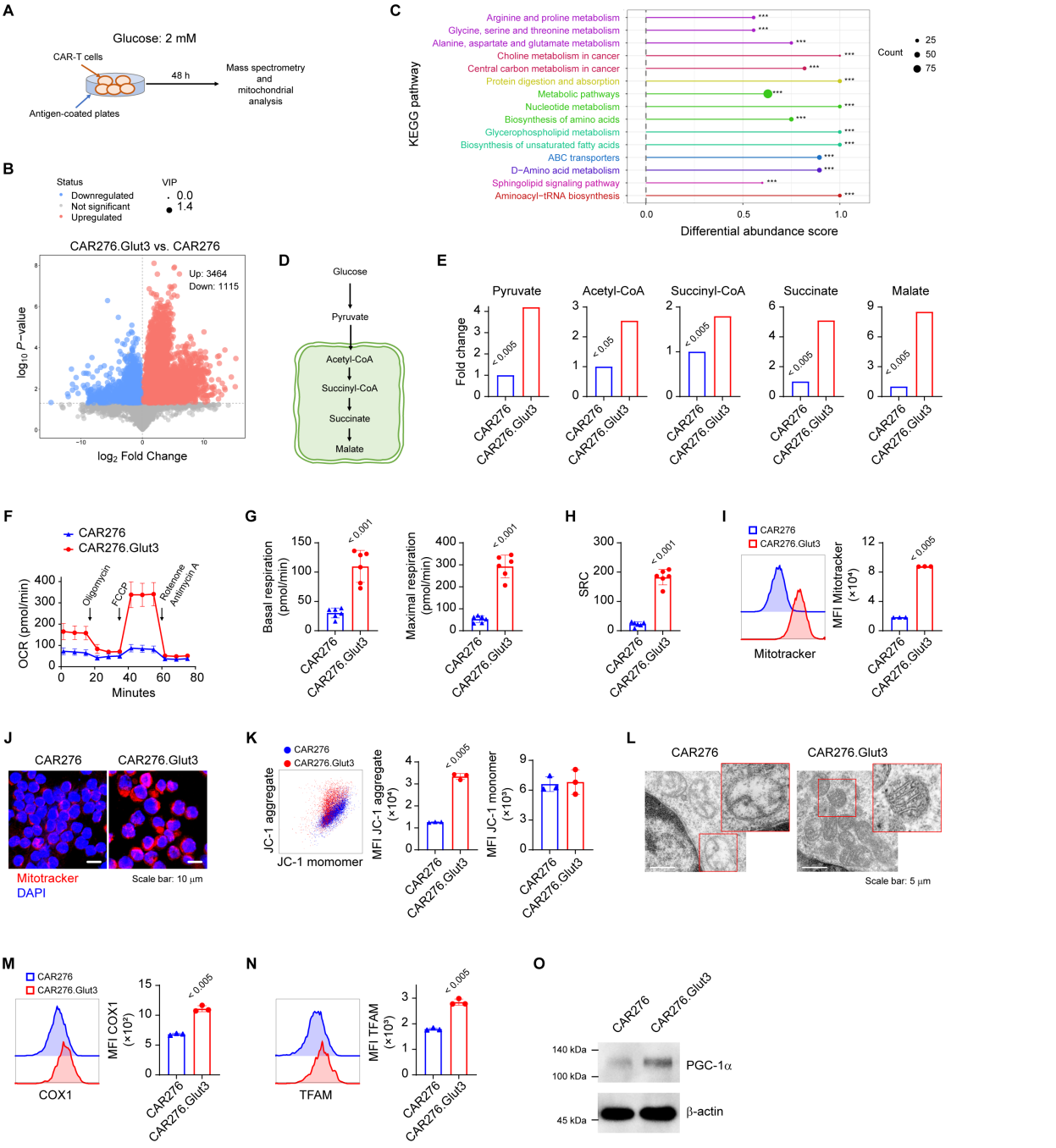

Figure 4 Glut3 overexpression improves mitochondrial fitness in chimeric antigen receptor T (CAR-T) cells. (A) Schematic depiction of sample preparation for metabolomics detection. CAR-T cells were activated by plate-coated antigens for 48 hours in 2 mM glucose. Then, CAR-T cells were collected and subjected to mass spectrometry analysis or mitochondrial analysis. (B) Volcano plot of differential metabolites in CAR276-T and CAR276.Glut3-T cells. Red dots represent the metabolites significantly upregulated in CAR276.Glut3-T cells. Blue dots represent the metabolites significantly downregulated in CAR276. Glut3-T cells. (C) KEGG pathway analysis of the differential metabolites in CAR276-T and CAR276.Glut3-T cells. (D) Key metabolites in central carbon metabolism in cancer pathways. (E) Changes in intracellular levels of the indicated metabolites in CAR276.Glut3-T cells to CAR276-T cells by mass spectrometry detection. (F–H) CAR-T cells were activated for 48 hours in 2 mM glucose and then their oxygen consumption rates (OCRs) were measured by seahorse energy detector (F). Subsequently, basal respiration, maximal respiration (G), and SRC (H) were determined. (I–J) CAR-T cells were activated for 48 hours in 2 mM glucose and stained with mitotracker dyes and subjected to FACS (I) and fluorescence microscopy detection (J), respectively. (K) CAR-T cells activated for 48 hours in 2 mM glucose were stained with JC-1 dyes and subjected to FACS analysis. (L) CAR-T cells activated for 48 hours in 2 mM glucose were collected and observed under electron microscopy. (M–O) CAR-T cells activated for 48 hours in 2 mM glucose were subjected to FACS or Western blot to detect COX1 (M), TFAM (N), and PGC-1a (O). CAR-T cells from four donors were subjected to metabolomic analysis, and their detection data were analyzed together. In the mitochondrial analysis (F–L), experiments were independently performed three times, and the results from one representative experiment are displayed. Two-tailed unpaired t-test for G-I, K, M, and N.

The TCA cycle occurs in mitochondria and can reflect the status of mitochondria.[28] Hence, we checked mitochondrial function by measuring the oxygen consumption rate, a surrogate for mitochondrial function status.[29] Compared with control CAR-T cells, CAR.Glut3-T cells showed increased basal and maximal respiration rates (figure 4F,G). In particular, CAR.Glut3-T cells had greater spare respiratory capacity, indicating enhanced mitochondrial function (figure 4H). It is reasonable to conclude that Glut3 overexpression improves the mitochondrial activity of CAR-T cells under conditions of low glucose. MitoTracker dye staining demonstrated that the number of mitochondria was increased in CAR.Glut3-T cells (figure 4I,J). Furthermore, more aggregated JC-1 was detected in CAR.Glut3-T cells, suggesting improved mitochondrial function in individual cells (figure 4K). Transmission electron microscopy revealed that both the quantity and morphology of mitochondria improved in CAR.Glut3-T cells (figure 4L). These results indicate that Glut3 overexpression promotes both the mass and fitness of mitochondria to increase their function in CAR-T cells activated under glucose restriction stress. We also assessed the expression of key proteins related to mitochondrial mass and function. As shown in figure 4M, cytochrome c oxidase subunit 1 (COX1), an indicator of mitochondrial mass,[12 30] was elevated in CAR.Glut3-T cells. Mitochondrial biogenesis and function-related proteins, mitochondrial transcription factor A (TFAM) and peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1a),[12 31] were upregulated in CAR.Glut3-T cells (figure 4N,O). Overall, these results indicate that Glut3 overexpression improves mitochondrial function of CAR-T cells under low glucose conditions.

CAR.Glut3-T cells had better tumoricidal activity and persistence in vivo

Then the antitumor potentials of CAR-T cells were evaluated in vivo. At first, the extracellular glucose levels within xenograft tumor tissues were detected (online supplemental figure S7A). In agreement with the clinical settings, the extracellular glucose in tumors was sharply decreased around or below 2 mM (online supplemental figure S7B). In Panc-1 xenograft tumors (figure 5A), CAR.Glut3-T cells induced tumor regression in all mice (figure 5B,C). In contrast, conventional CAR-T cells delayed tumor growth in most mice but failed to control tumor growth finally (figure 5B,C). Consequently, CAR.Glut3-T cells markedly improved mouse survival (figure 5D). CAR.Glut3-T cells showed an improved glucose uptake capacity (figure 5E), and elevated infiltration (figure 5F,G), activation (figure 5H), and effector cytokine production (figure 5I,J) within tumor tissue. Further assays showed that exhaustion-related molecules (TIM-3, LAG-3, TOX) were increased in conventional CAR-T cells while stemness marker TCF-1 was increased in CAR.Glut3-T cells (online supplemental figure S7C–E). Following that, the antitumor efficacy of different CAR-T cells was compared in recurrent models (figure 5K). CAR276-T or CAR276.Glut3-T cells were infused 7 days after tumor cell incubation, to achieve complete tumor regression 7–14 days post-CAR-T cells infusion (data not shown). At 28 days post-CAR-T cell infusion (indicated as day −1), mice maintained complete tumor regression (figure 5L). FACS analysis showed that both CAR276-T cells and CAR276.Glut3-T cells were present in the blood of mice on day −1 (figure 5O). However, the ratio and number of CAR276.Glut3-T cells increased (figure 5O,P). The next day, the mice were inoculated with *FFluc*-expressing Panc-1 cells again (figure 5L). In rechallenged mice, CAR276.Glut3-T cells maintained potent antitumor activity and induced tumor regression in all mice (figure 5L–N). In contrast, conventional CAR276-T cells were unable to efficiently suppress tumor growth (figure 5L–N). As expected, CAR276.Glut3-T cells markedly extended mouse survival compared with CAR276-T cells (figure 5N). These results indicated that the Glut3 modification improved tumoricidal activity and persistence of CAR-T cells in vivo.

CAR.Glut3-T cells achieved better antitumor activity in multiple tumor models

In advanced tumor models (figure 6A), conventional CAR276-T cells moderately suppressed tumor growth (figure 6B,C). CAR276.Glut3-T cells delayed the progression of advanced tumors more efficiently and eventually induced complete tumor regression in more than half of the mice (figure 6B,C). Consistently, CAR276.Glut3-T cells extended the survival of mice (figure 6D).

The antitumor efficacy of CAR.Glut3-T cells was evaluated in a metastatic tumor model (figure 6E). As expected, fewer nodules were observed in the lungs of mice treated with CAR276.Glut3-T cells (figure 6F,G), indicating that CAR276.Glut3-T cells maintain a potent antitumor potential in metastatic tumors. The KYSE150 xenograft tumor model was established and treated (online supplemental figure S7F). As shown in online supplemental figure S7G–I, CAR276.Glut3-T cells demonstrated more potent tumoricidal activity in this esophageal cancer xenograft model. CAR276.Glut3-T cells induced tumor regression and extended mouse survival more efficiently than conventional CAR276-T cells in models of esophageal carcinoma (online supplemental figure S7G–I). To determine whether the increased antitumor effects of Glut3 were antigen-independent, mesothelin-targeting CAR-T cells were generated. Under glucose-limited conditions, CARmeso.Glut3-T cells killed H322 lung cancer cells in vitro (figure 6H). H322 xenograft tumor models were established, and mice were treated with CAR-T cells (figure 6I). Compared with CARmeso-T cells, CARmeso.Glut3-T cells suppressed tumor growth more efficiently and extended mouse survival (figure 6J–L). Immunodeficient mice were systematically implanted with H322 cells and treated with CAR-T cells (figure 6M). Fewer tumor nodules were observed in the lungs of mice treated with CARmeso.Glut3-T cells than in those treated with UTD or CARmeso-T cells (figure 6M–O). Additionally, the impact of Glut3

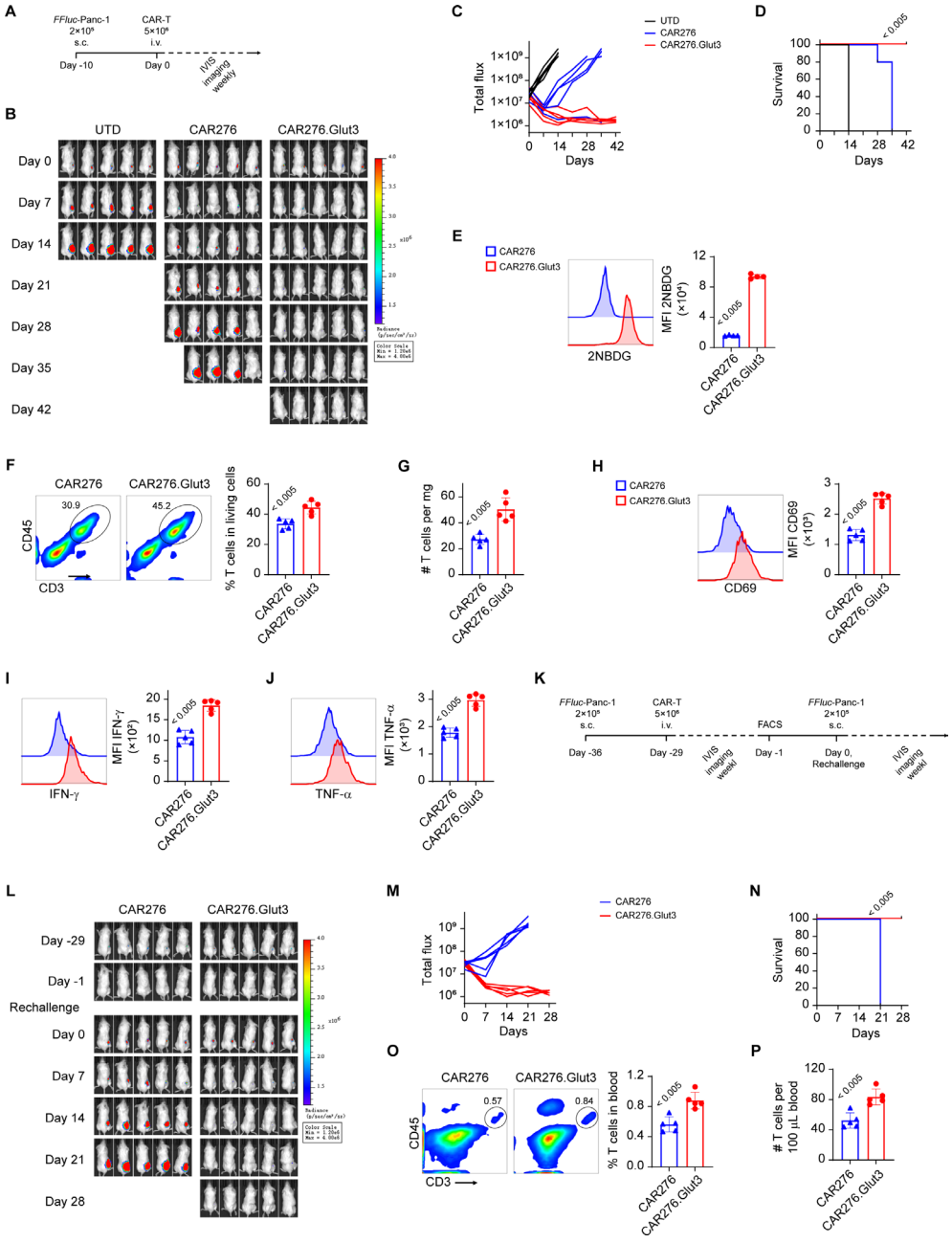

Figure 5 Glut3 overexpression improves antitumor potentials of chimeric antigen receptor T (CAR-T) cells in vivo. (A–D) CAR-T cell therapy in Panc-1 xenograft tumor model. 10 days post *FFluc*-Panc-1 cells were inoculated into immune-deficient NTG mice subcutaneously ((n=5 per group), CAR-T cells were administered through tail veins (A). Tumor growth was monitored using BLI imaging weekly after CAR-T cell infusion. Tumor BLI images (B) and individual BLI kinetics (C) are displayed. Kaplan-Meier survival curve of mice treated with indicated CAR-T cells (D). (E) Analysis of 2NBDG uptake by tumor-infiltrating CAR-T cells (n=5 per group). As depicted in A, NTG mice with established Panc-1 tumors were treated with CAR-T cells. Ten days post CAR-T cell infusion, 2NBDG was injected through tail veins and tumors were harvested 30 min later. 2NBDG uptake by intratumoral CAR-T cells was analyzed by flow-activated cell sorting (FACS). (F–J) Functional analysis of tumor-infiltrating CAR-T cells (n=5 per group). NTG mice with established Panc-1 tumors were treated with CAR-T cells according to the procedure described in A. 10 days after CAR-T cells injection, tumors were harvested. Infiltration and numbers of infiltrating CAR-T cells were determined by FACS detection (F) and counting (G). Additionally, CD69 (H), IFN-g (I), and TNF-a (J) of intratumoral CAR-T cells were detected by FACS. (K–N) CAR-T cell therapy in relapsed tumor model. CAR-T cells induced complete regression after first injection of tumor cells in NTG mice. Then *FFluc*-Panc-1 cells were inoculated subcutaneously into mice maintaining tumor regression (n=5 each group; K). Post rechallenge, tumor growths were monitored with bioluminescence (BLI) imaging weekly. Tumor BLI images (L) and individual BLI kinetics (M) are shown. Kaplan-Meier survival curve of mice receiving listed CAR-T cell infusion (N). (O and P) Persistence of CAR-T cells in vivo (n=5 per group). As depicted in K, NTG mice were inoculated with Panc-1 tumor cells, followed by CAR T-cell therapy. At 28 days post-CAR-T cell infusion, blood samples were collected from the tail veins of mice maintaining complete regression. The ratios and numbers of circulating CAR-T cells were determined using FACS (O) and counting (P), respectively. Two tailed unpaired t-test in E–J, O, P.

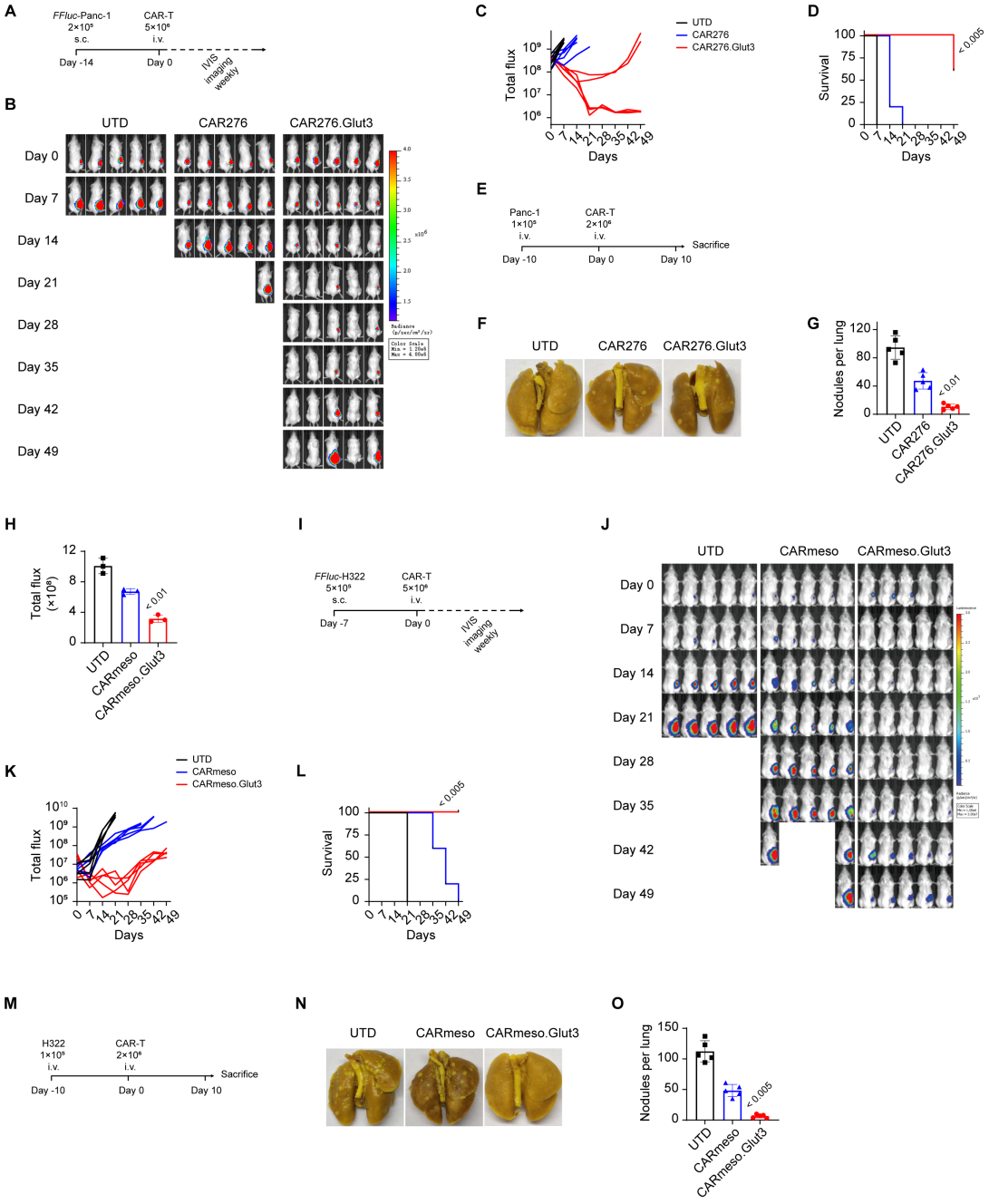

Figure 6 Glut3 overexpression improves antitumor potentials of chimeric antigen receptor T (CAR-T) cells in multiple models. (A–D) Evaluation of CAR-T cell therapy in an advanced tumor model: 14 days post-*FFluc*-Panc-1 cell inoculation, NTG mice were given CAR-T cells via the tail veins (n=5 per group; A). Tumor growth was monitored using bioluminescence (BLI) imaging weekly after CAR-T cell infusion. Tumor BLI images (B) and individual BLI kinetics (C) are displayed. Kaplan-Meier survival curve of mice treated with indicated CAR-T cells (D). (E–G) CAR-T cell therapy in metastatic tumor model: Panc-1 cells were injected into NTG mice via tail veins and CAR-T cells were administered 10 days later (n=5 per group; E). 10 days post-CAR-T cells infusion, mice were sacrificed. Lungs were isolated, stained, and nodules were counted. Representative lung photos (F). Numbers of tumor nodules in lungs from mice receiving different treatments (G). (H) Co-culture assay. Mesothelin-specific CAR-T cells were co-cultured with *FFluc*+H322 cells at E:T=1:1 in media having 2 mM glucose for 48 hours. Tumor cells bioluminescence (BLI) was detected with IVIS imaging. (I–L) CARmeso-T cell therapy in H322 xenograft tumor model: Seven days post *FFluc*-H322 cells were inoculated into NTG mice subcutaneously, CAR-T cells were given through tail veins (n=5 per group; I). Tumor growth was monitored using BLI imaging weekly after CAR-T cell infusion. Tumor BLI images (J) and individual BLI kinetics (K) are displayed. Kaplan-Meier survival curves of mice receiving the indicated CAR-T cells infusion (L). (M-O) CARmeso-T cell therapy in metastatic tumor model: H322 cells were injected into NTG mice via tail veins and CAR-T cells were administered 10 days later (n=5 per group; M). Ten days post-CAR-T cell infusion, mice were euthanized. Lungs were stained and nodules were counted. Representative lung photos (N). Numbers of tumor nodules in lungs from the indicated CAR-T cell-treated mice (O). One-way ANOVA with Tukey's multiple comparison test was used for G, H, and O. ANOVA, analysis of variance. IVIS, in vivo imaging system.

overexpression in CD28-costimualated CAR-T cells (CAR.28z-T) cells was examined (online supplemental figure S8). Glut3-overexpressing enhanced the tumoricidal potential of CAR.28z-T cells, which exhibited specificity for recognition of CD276 or mesothelin (online supplemental figure S8B, D). These results suggest that overexpression of Glut3 improves the antitumor potential of CAR-T cells against solid tumors.

Glut3 overexpression improved CAR-T cell function in the syngenetic tumor model

Myeloid cells also consume a large amount of glucose within the tumor microenvironment.[11] To study the antitumor functions of CAR-T cells overexpressing Glut3 in a more clinically related situation, immunocompetent mice-bearing syngenetic tumors were used in the syngenetic tumor model. Mouse CAR vectors that recognize the extracellular region of human CD276 were synthesized. For convenient detection, FLAG and truncated NGFR (tNGFR) tags were inserted on mouse CAR vectors (online supplemental figure S9A). Next, mouse CAR-T (mCAR-T) cells were generated (online supplemental figure S9B). Mouse LLC tumor cells expressing human extracellular parts of CD276 were subcutaneously inoculated into immune-competent C57 mice and mCAR-T cells were infused 7 days later (figure 7A). Compared with mCAR276-T cells, mCAR276.Glut3-T cells markedly suppressed LLC tumor growth and increased mouse survival (figure 7B,C). Within tumor tissues, infiltration of mCAR276.Glut3-T cells was more intense than that of conventional mCAR276-T cells (figure 7D). FACS analysis demonstrated that mCAR276.Glut3-T cells had intensified 2NBDG uptake (figure 7E), suggesting upregulated glucose uptake within tumor tissues. The FACS assay further demonstrated that the number of infiltrated mCAR276.Glut3-T cells outperformed mCAR276 -T cells (figure 7F,G). Furthermore, mCAR276.Glut3-T cells displayed increased expression of the activation marker CD69 and enhanced effector IFN-γ and TNF-α cytokine production within tumors (figure 7H–J).

Enhanced glucose uptake and glycolytic activity promote the function of pro-inflammatory T cells, which can lead to inflammatory diseases.[32] In our model, Glut3-overexpressing CAR-T cells showed enhanced antitumor capacity within malignant lesions. It is important to determine whether Glut3-overexpressing CAR-T cells exhibit systemic toxicity beyond tumor lesions. Therefore, we evaluated the safety of Glut3-overexperssing CAR-T cell treatment. As shown in figure 7K,L, treatment with mCAR276.Glut3-T cell did not result in weight loss or abnormal inflammatory responses in mice, suggesting a good safety profile of Glut3-overexpressing CAR-T cells. These syngenetic models indicate that Glut3 overexpression confers CAR-T cells with upregulated glucose uptake capacity and antitumor potential in immune-competent settings.

DISCUSSION

Glucose restriction is common in solid tumor tissues. It is well established that glucose insufficiency can induce a deficiency in T cell function. CAR-T cells face the same obstacles as those of solid tumor tissues. However, the effect of glucose limitation on CAR-T cells has not been evaluated to date. In this study, we discovered that a low glucose supply markedly decreased CAR-T cell activation, effector function, and cell expansion. We also developed an efficient approach for CAR-T cells to overcome glucose restriction and functional deficiencies in the TME.

Metabolic status can influence the antitumor potential of T cells and CAR-T cells.[5 33] Both naïve and memory T cells markedly increase glucose metabolism,[19 34] which in turn translates to an increased glucose metabolism requiring increased glucose uptake. When glucose uptake is restricted, T-cell activation, effector function, and survival are suppressed.[8 19 35] Consistently, our work shows that glucose levels positively determined the antitumor properties of CAR-T cells, including activation levels, effector functions, and expanding potential. Based on these observations, it is critical to overcome the poor glucose uptake of CAR-T cells to support optimal antitumor effects within solid tumors.

Glucose uptake is mediated by Gluts. T cells mainly express Glut1 and upregulate Glut1 expression after activation.[19] When glucose is sufficient, overexpression of Glut1 can augment the metabolic fitness and proinflammatory functions of CAR-T cells.[36] In our study, it was also noticed that the Glut1-overexpressing CAR-T cells had advantages in proliferation and memory differentiation during expansion in media having around 10 mM glucose. Nonetheless, Glut1 has an affinity for glucose at 3–7 mM[20] and less efficiently transports glucose in the solid tumor environment, where the glucose levels are much lower.[11 21 22] Although Glut1 is upregulated in activated T cells[36 37] and can retain glucose transport ability within tumors,[37] neither of the conventional and Glut1-overexpressed T cells can uptake enough glucose to fully exert tumoricidal funcitons.[10 37] Similarly, our work shows that the conventional and the Glut1-overexpressing CAR-T cells both expressed high levels of Glut1 but have significantly impaired functions in media having 2 mM glucose, which represents a relatively high level of environmental glucose in solid tumors. Hence, it is very critical to improve the ability of CAR-T cells to uptake low levels of glucose. The Glut3 transporter has a much higher affinity for glucose (1.4 mM) than Glut1.[20] Glut3 is predominantly expressed in highly glucose-dependent tissues and is efficient in transporting low levels of glucose into cells,[38] making it more suitable for glucose transport than Glut1 in the tumor microenvironment. However, T cells express low levels of Glut3 even after activation.[18 19] Our study demonstrates that only a small proportion of human CAR-T cells express Glut3. Therefore, we overexpressed Glut3 in CAR-T cells. These Glut3-overexressing CAR-T cells achieved enhanced glucose uptake capacity and displayed more effective antitumor potential under

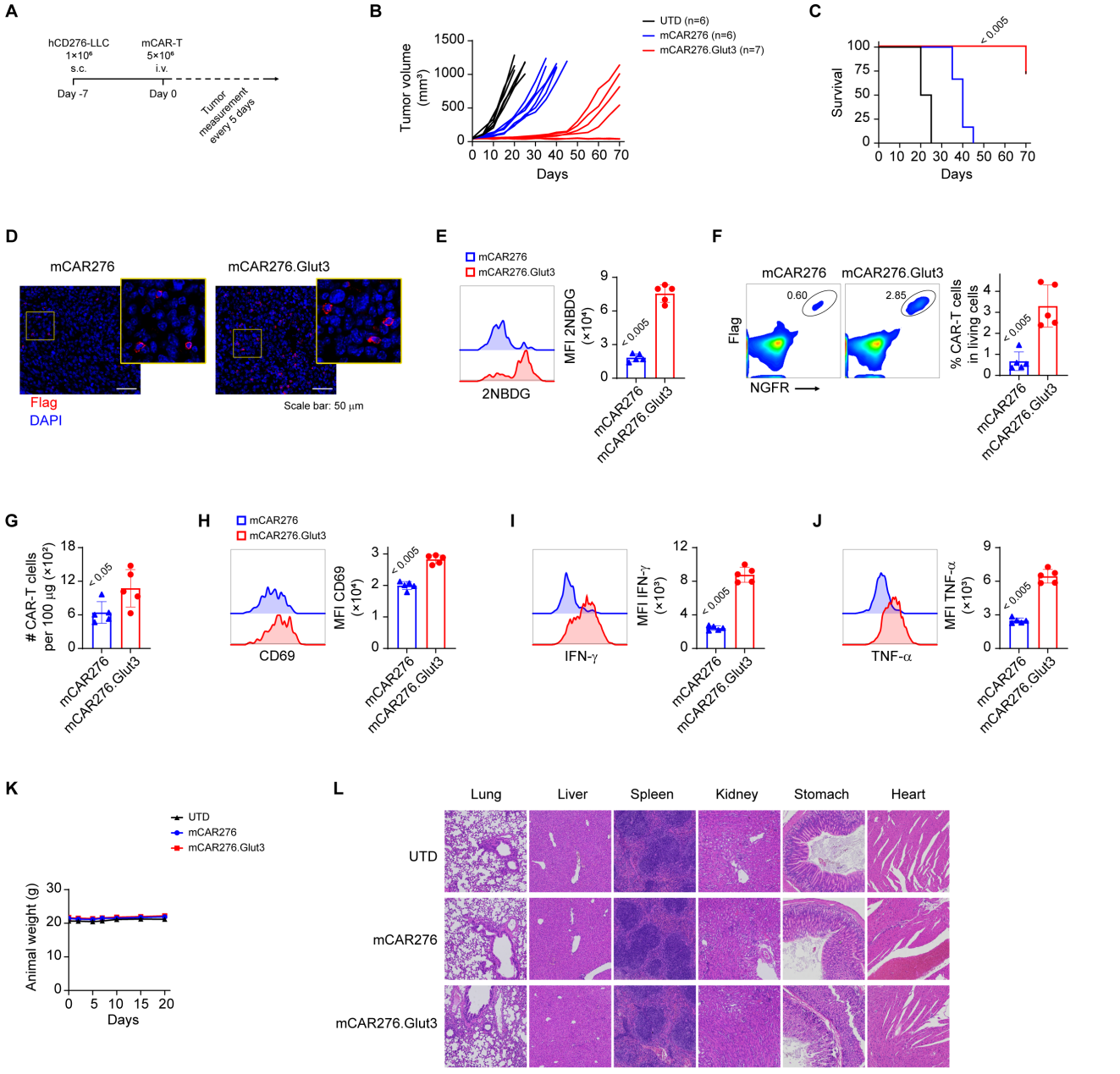

Figure 7 Glut3 overexpression improves anti-tumor potentials of chimeric antigen receptor T (CAR-T) cells in syngenetic tumor model. (A–C) CAR-T cell therapy in syngenetic tumor model 7 days after immune-proficient C57 mice were inoculated with LLC tumor cells expressing truncated human CD276 subcutaneously, mouse CAR-T cells recognizing human CD276 were injected via tail veins (Six mice in UTD or mCAR276 groups; 7 mice in mCAR276.Glut3 group; A). Tumor growth was measured twice per week after CAR-T cell infusion. Individual tumor volumes (B). Kaplan-Meier survival curve of mice treated with indicated CAR-T cells (C). (D) Analysis of tumor infiltration of CAR-T cells (n=3 for each group). As shown in A, C57 mice with established hCD276-LLC tumors were treated with mouse CAR-T cells. 10 days post-CAR-T cells infusion, tumors were harvested and sectioned. CAR-T cell infiltration was visualized by Flag staining. (E) Analysis of 2NBDG uptake by tumor-infiltrating CAR-T cells (n=5 per group). As depicted in A, C57 mice with established hCD276-LLC tumors were treated with mouse CAR-T cells. Ten days post-CAR-T cell infusion, 2NBDG were injected through tail veins and tumors were harvested 30 min later. 2NBDG uptake by intratumoral CAR-T cells was analyzed with flow-activated cell sorting (FACS). (F–J) Infiltration and function analysis of tumor-infiltrating CAR-T cells (n=5 per group): C57 mice with established hCD276-LLC tumors were treated with mouse CAR-T cells as described in A. Ten days after CAR-T cell injection, tumors were harvested. Infiltration and numbers of infiltrating CAR-T cells were determined by FACS (F) and cell counting (G). Additionally, CD69 (H), IFN-g (I), and TNF-a (J) of intratumoral CAR-T cells were detected by FACS. (K and L) Mouse weights and HE staining of major organs after CAR T-cell therapy (n=5 per group): C57 mice with established hCD276-LLC tumors were treated with mouse CAR-T cells, as described in A. After CAR-T cell infusion, mouse weights were measured every 5 days (K). Mice were sacrificed 21 days post-CAR-T cell infusion, and the major organs were harvested and subjected to HE staining. Representative images of the HE-stained organs are shown (L). Two-tailed unpaired t-test for G–I and K in E–J. Two-way ANOVA with Tukey's multiple comparison test was performed in K. ANOVA, analysis of variance. LLC, Lewis lung carcinoma.

glucose-restricted conditions in both in vitro and in vivo assays. Consistent with our findings, Glut3 overexpression improved the antitumor potential of antigen-specific T cells,[39] whose capacity was not promoted by Glut1-overexpresson in a solid tumor model.[37] Additionally, Glut10 has a very high glucose affinity.[40] But the glucose-uptake capacity of Glut10 is largely restricted by lactic acid, which is abundant in tumor microenvironment.[40] In contrast, Glut3 is not affected by environmental factors including lactic acid as seen in our observations and other's study.[39] Hence, Glut3 is more suitable for improving CAR-T cell therapy on solid tumors.

The PI3K/Akt pathway is important for the activation, proliferation, and persistence of native T and CAR-T cells.[27 41] It has been shown that CAR-T cells with enhanced activation of the PI3K/Akt pathway have better effector functions, proliferation, and persistence.[41 42] Glycolysis can promote the PI3K/Akt pathway and, therefore, can improve the activation and proliferation of T cells.[7 9] It was observed that Glut3-overexpressing CAR-T cells had enhanced glycolysis and activation of the PI3K/Akt pathway compared with conventional CAR-T cells. Correspondingly, Glut3-overexpressing CAR-T cells overexpressing Glut3 showed increased activation and expansion compared with their non-overexpressing counterparts. Consistently, enhanced glycolysis is closely related to enhanced PI3K/Akt pathway activation, improved effector functions, and persistent expansion of CAR-T cells.[17 36]

Mitochondria play a central role in the regulation of T-cell function and fate.[43] Similarly, mitochondrial fitness determines the antitumor potential of CAR-T cells.[31] When mitochondrial mass and function increase, CAR-T cells show extended persistence and increased antitumor potential.[12 17 30] Accordingly, our study shows that mitochondrial fitness, including mass and function, is improved in Glut3-overexpressing CAR-T cells, which enhances antitumor potential after chronic stimulation. Acetyl-CoA can increase mitochondrial function and antitumor capacity of T cells.[44] In our study, we observed a marked increase in acetyl-CoA in Glut3-overpressing CAR-T cells. In activated T cells, intracellular levels of acetyl-CoA are determined by glucose supply and glycolytic activity.[44 45] Consistently, our study showed that Glut3-overexpressing CAR-T cells with enhanced glucose uptake and glycolysis produced higher amounts of acetyl-CoA.

PGC-1a is the key regulator of mitochondrial mass and functions.[31 46] It has been shown that PGC-1a can improve the effectiveness of engineered T cell therapy.[46 47] In agreement, it was noticed that Glut3-overexpressing CAR-T cells had increased PGC-1a expression and antitumor efficacy. Interestingly, PI3K-Akt pathway and PGC-1a were both augmented in activated Glut3-overexpressing CAR-T cells. PI3K-Akt pathway can suppress PGC-1a and then impair mitochondrial functions in T cells.[48] But the concurrent augmentation of PI3K/Akt pathway and mitochondrial fitness, which reflect PGC-1a levels, are frequently noted in CAR-T cells.[17 30] This discrepancy is probably due to the activating intensities of PI3K-Akt pathway. The high levels of PI3K-Akt pathway activation can increase PGC-1a.[49 50] Glut3-overexpressing CAR-T cells had higher levels of activated Akt and the downstream molecules than conventional CAR-T cells. Hence, the increased PI3K/Akt pathway might contribute to PGC-1a upregulation. But the exact mechanisms remain explored.

The present study has some limitations. First, higher glucose metabolism induces the upregulation of T-cell function and increases the expression of immune-inhibitory receptors, such as PD-1, which in turn can inhibit glycolysis[25] and is critical for Glut3 to improve CAR-T cell function. It is worth investigating the immunosuppressive effects of checkpoint receptors and developing combination regimens for more effective therapies. Second, human tumors are much larger than those in this study and confer more severe nutrient deprivation, including much lower glucose. Under such conditions, Glut3 may lose its efficiency in transporting glucose into T cells. Gluts with a stronger affinity for glucose can be artificially constructed and exploited in the future.

In conclusion, glucose deprivation induces T cell dysfunction, which represents a critical obstacle in CAR-T cell therapy. Here, we developed an efficient strategy to improve the uptake of low levels of extracellular glucose and to enhance the tumoricidal potential of CAR-T cells against solid tumors.

MATERIALS AND METHODS

Animal experiments

Female NTG or C57BL/6J mice, aged 6–8 weeks, were purchased from SPFbiotech (Beijing, China). Detailed procedures are provided in online supplemental file 2.

Statistical analysis

Data are presented as the mean±SD. P values <0.05 were considered statistically significant. Statistical analyses and graph generation were performed using Prism V.9 software (GraphPad, San Diego, California, USA).

Contributors FL conceived the study; FL, YuZ, and WH designed the study and analyzed the data; WH, FL, and YL performed experiments; SL, SW, CS, YuZ, and HW assisted with experiments and analyses; FL and WH wrote the manuscript; FL and YuZ revised the manuscript. YiZ is the guarantor in the contributorship statement.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the Ethics Review Committee of the First Affiliated Hospital of Zhengzhou University (2019-KY-258). Participants gave informed consent to participate in the study before taking part. All animal experiments were conducted under the guidance of the Ethics Committee of Zhengzhou University (approval n. ZZU-LAC20210702[(11)]).

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Data availability statement Data are available on reasonable request. The data for this study can be obtained by contacting the corresponding authors on reasonable request.

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