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Selective glutamine metabolism inhibition in tumor cells improves antitumor T lymphocyte activity in triple-negative breast cancer

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Rapidly proliferating tumor and immune cells need metabolic programs that support energy and biomass production. The amino acid glutamine is consumed by effector T cells and glutamine-addicted triple-negative breast cancer (TNBC) cells, suggesting that a metabolic competition for glutamine may exist within the tumor microenvironment, potentially serving as a therapeutic intervention strategy. Here, we report that there is an inverse correlation between glutamine metabolic genes and markers of T cell–mediated cytotoxicity in human basal-like breast cancer (BLBC) patient data sets, with increased glutamine metabolism and decreased T cell cytotoxicity associated with poor survival. We found that tumor cell–specific loss of glutaminase (GLS), a key enzyme for glutamine metabolism, improved antitumor T cell activation in both a spontaneous mouse TNBC model and orthotopic grafts. The glutamine transporter inhibitor V-9302 selectively blocked glutamine uptake by TNBC cells but not CD8+ T cells, driving synthesis of glutathione, a major cellular antioxidant, to improve CD8+ T cell effector function. We propose a "glutamine steal" scenario, in which cancer cells deprive tumor-infiltrating lymphocytes of needed glutamine, thus impairing antitumor immune responses. Therefore, tumor-selective targeting of glutamine metabolism may be a promising therapeutic strategy in TNBC.

Introduction

Rapidly dividing cells display unique metabolic traits that support their enhanced energy requirements and ever-increasing biomass (amino acids, nucleotides, lipids, etc.) synthesis to enable continued proliferation (1–9). The ability of cancer cells to hijack these metabolic processes is a known hallmark of cancer (10). Although glucose metabolism has received substantial attention, the role of amino acid metabolism in tumorigenesis and tumor progression has started to be explored more recently (11–14). Some cancer types are particularly dependent on metabolism of the amino acid glutamine for tumor cell survival and/or proliferation. For example, triple-negative breast cancers (TNBCs), which do not express estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor (EGF) receptor 2 (HER2), are particularly "addicted" to glutamine as compared with other breast cancer subtypes (15, 16). TNBCs often express high levels of ASCT2, a

glutamine transporter, and glutaminase (GLS), the enzyme that catalyzes the rate-limiting step in glutaminolysis, the conversion of glutamine into glutamate (17–19). Unlike other breast cancer subtypes, TNBCs do not have the benefit of molecularly targeted therapies, relying primarily on chemotherapeutic regimens. However, a growing body of evidence demonstrates that glutamine is required for TNBC tumor cell survival (17–20), suggesting that glutamine metabolism may represent a therapeutic target in TNBC.

Like tumor cells, activation of T lymphocytes increases glutamine uptake and metabolism to support mitochondrial anaplerosis, nucleotide synthesis, amino acid production, and redox balance (21). Ligation of CD3 and CD28 on T cells, an event that drives T cell expansion and activation, leads to upregulation of glutamine transporters such as ASCT2, SNAT1, and SNAT2 to facilitate glutamine uptake by T cells (22–24). Further, glutamine starvation of T cells significantly hinders T cell proliferation and cytokine production (22), illustrating the critical nature of glutamine metabolism for T cell–mediated immune responses. Although ASCT2 loss from T cells did not appear to limit T cell proliferation or cytotoxicity in the acute setting of viral infection in vivo (25), chronic GLS inhibition blocked conversion of glutamine to glutamate and reduced long-term CAR-T cell responses in an

in vivo tumor model (26). Of note, immunosuppressive T regulatory cells (Tregs) are able to maintain reserve respiratory capacity and thrive even in the absence of glutamine (27). Together, these studies suggest that glutamine supply to and metabolism in T cells influence the nature and potency of immune responses, but also suggest that glutamine transporter blockade may not fully impede glutamine uptake into T cells.

The increased metabolic demands of tumor cells and activated T lymphocytes may introduce competition for glutamine within the tumor microenvironment (8, 28–30), creating a scenario in which tumor cells out-compete T cells for local glutamine and thereby alter the characteristics of the tumor-infiltrating lymphocytes (TILs). Thus, in this scenario the nutrient consumption would both promote proliferation and survival of tumor cells and simultaneously limit the capacity for T cell–mediated antitumor immunity, similar to observations with glucose (31, 32). Accordingly, we hypothesized that glutamine-dependent tumors, such as TNBCs, might benefit therapeutically from inhibition of glutamine metabolism, improving antitumor T cell responses by reversing a tumor "glutamine steal" phenomenon.

In this report, we analyzed human clinical basal-like breast cancer (BLBC) data sets and found an inverse relationship between glutamine metabolism and T cell cytotoxicity markers and a poor overall survival in patients whose tumors harbor both high glutamine metabolism and low T cell cytotoxicity signatures. Using spontaneous genetically engineered and orthotopically grafted mouse models of TNBC, we show that tumor cell–specific GLS loss increased tumor interstitial glutamine concentration and intratumoral T cell infiltration and activity. Pharmacological inhibition of glutamine uptake using the glutamine transporter inhibitor, V-9302, blocked glutamine uptake in tumor cells but not in T cells, which adapted through compensatory upregulation of another glutamine transporter, resulting in superior T cell responses within tumors while diminishing tumor growth. Together, these data indicate that tumor-selective blockade of glutamine uptake may be a feasible, molecularly targeted approach to combat TNBC, providing a 2-pronged attack that boosts antitumor immune responses while concurrently crippling tumor cell metabolism.

Results

Expression of glutamine metabolism genes inversely correlate with cytotoxic gene signatures in human BLBC. To examine if tumor cell glutamine metabolism may negatively affect antitumor immunity in TNBC, we analyzed BLBC expression data sets curated by The Cancer Genome Atlas (TCGA). BLBCs are the molecularly defined breast cancer subtype characterizing most TNBC specimens (33). Gene expression values for genes encoding glutamine-utilizing enzymes (*GLS*, *ASNS*, and *GFPT1*) as well as glutamine transporters (ASCT2/*SLC1A5*, SNAT1/*SLC38A1*, SNAT2/*SLC38A2*, and ATB⁰,⁺/*SLC6A14*) were used to generate a glutamine metabolism gene signature (Figure 1A). To avoid a potentially confounding bias from more highly glycolytic tumor samples, a glycolysis signature consisting of expression values for the genes encoding glycolysis enzymes was used to identify those tumor samples with reduced glycolysis (Figure 1A). The resulting "glutamine metabolism high" subset of patient samples exhibited a significantly stronger expression profile for glutamine metabolism than glycol-

ysis, while all others are classified as having "mixed metabolism." A CTL gene signature was generated using 4 genes often associated with increased CD8⁺ T cell cytotoxic activity, *GZMA*, *GZMB*, *PRF1*, and *IFNG*. Correlation analyses of the glutamine metabolism high samples revealed a strong negative correlation between the glutamine metabolism and CTL gene signatures (Figure 1B, black). The same relationship between the 2 parameters was not observed in the mixed metabolism samples (Figure 1B, pink). Similarly, inverse correlations were observed between *GLS*, the rate-limiting enzyme in glutaminolysis, and the CTL gene signature (Figure 1C). Comparable trends between glutamine metabolism markers and individual markers of CTL expansion and effector function (*GZMA*, *GZMB*, *PRF1*, or *IFNG*) alone (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI140100DS1) were also observed, suggesting that a strong antithetical relationship exists between glutamine metabolism and cytotoxic factors in BLBC tumors.

To evaluate whether glutamine metabolism and CTL activity may influence TNBC patient survival, we stratified BLBC patients based on their tumor glutamine metabolism gene signature (GMGS) as well as their CTL gene signatures (Figure 1D). Patients were then classified into 4 categories: GMGS^LOW/CTL^HIGH, GMGS^LOW/CTL^LOW, GMGS^HIGH/CTL^HIGH, or GMGS^HIGH/CTL^LOW, and a Kaplan-Meier plot was used to display patient survival for each group (Figure 1D). Having high expression of glutamine metabolic genes combined with low CTL expression resulted in reduced overall survival as well as distant metastasis-free survival compared with other groups (Figure 1D and Supplemental Figure 1B). Furthermore, survival was significantly reduced when only assessing CTL gene expression with *GLS* alone (Supplemental Figure 1C). Together, these data suggest that high glutamine metabolism along with low cytotoxic potential of T cells within tumors may contribute to poorer outcomes in TNBC patients.

Loss of GLS in tumor cells reduces E0771 tumor growth and metastasis in a lymphocyte-dependent manner. Given that human TNBC cells have increased GLS and glutaminolysis and that T cells require glutamine metabolism for proper activation and function (17–19), we assessed the impact of cancer cell–specific glutamine metabolism on T lymphocytes within the tumor microenvironment using a mouse TNBC model. We used CRISPR-Cas9 technology in the murine TNBC line E0771 (33, 34) to generate clonal variants with GLS loss-of-function mutations (Supplemental Figure 2A), referred to here as E0771-GLS^KO. Intracellular glutamate, a measure of glutaminolysis, was significantly decreased in the E0771-GLS^KO clones (Supplemental Figure 2B), confirming decreased glutaminolysis in the E0771-GLS^KO cells. Next, wild-type (WT) or GLS^KO cells were orthotopically implanted into the inguinal mammary fat pads of female C57BL/6 mice. Tumors were detected in 100% of mice from each group; however, tumor growth was diminished substantially in E0771-GLS^KO tumors, as measured by tumor volume (Figure 2A) and tumor weight (Figure 2B). Immunohistochemical (IHC) analysis of tumors identified abundant GLS expression in E0771-WT tumors but not in E0771-GLS^KO tumors (Figure 2C). Ki67 was used as a measure of tumor cell proliferation, and despite the smaller size of E0771-GLS^KO tumors, Ki67 was detected at similar proportions in E0771-GLS^KO and E0771-WT tumors (Figure 2D), suggesting that
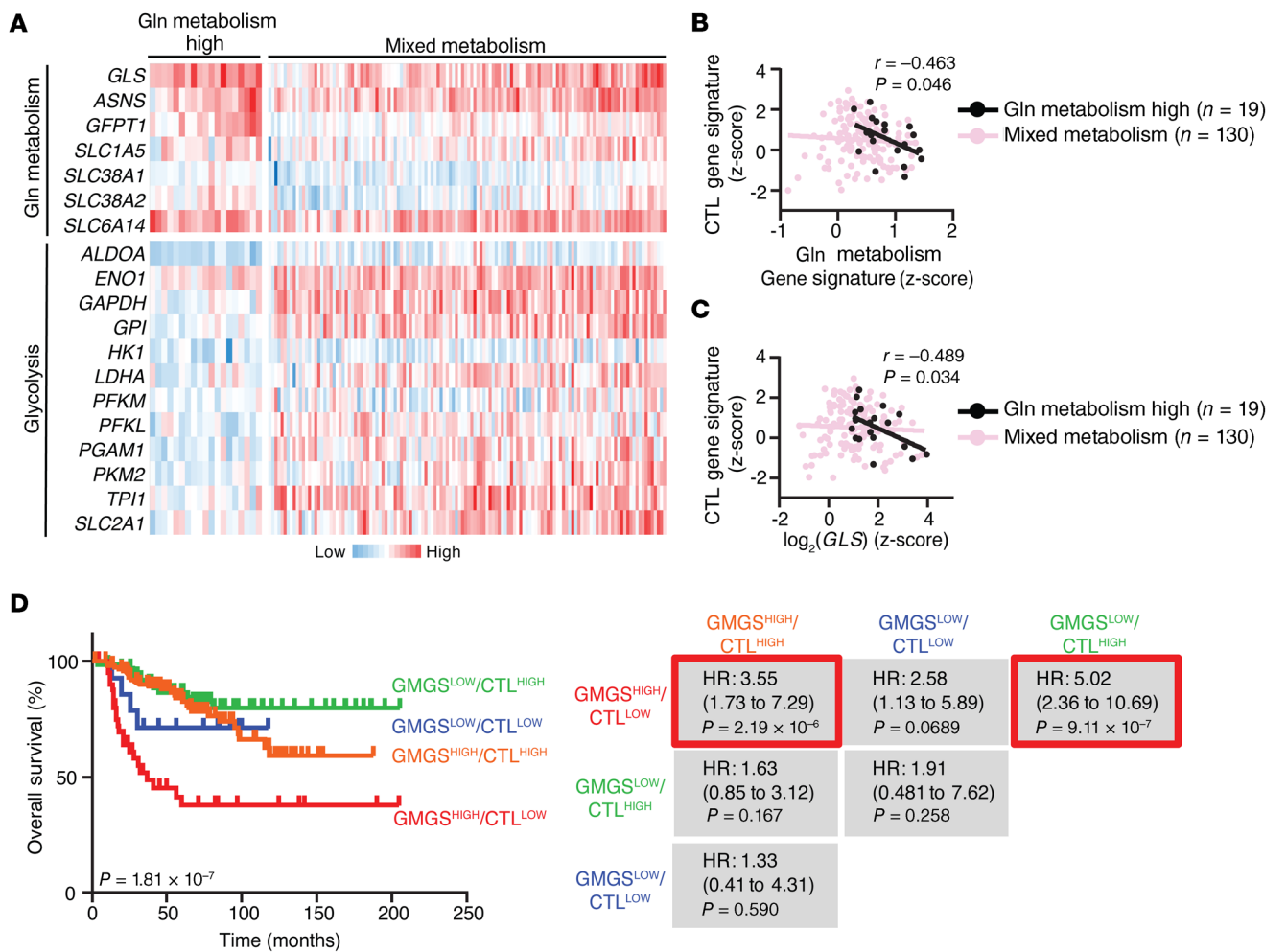
Figure 1. Glutamine metabolism inversely correlates with expression of T cell activation markers in glutamine metabolism high human basal-like breast cancer samples. (**A**–**C**) Analysis of mRNA expression ($\log_2$) z-scores in basal-like tumor samples from TCGA BRCA data set. The samples with the greatest glutamine metabolism gene signature (GMGS) combined with the lowest glycolysis gene signature are considered "glutamine metabolism high." All others are considered to have "mixed metabolism." (**A**) Expression levels of individual genes are displayed as a heatmap, with low expression in blue and high expression in red. (**B** and **C**) The CTL gene signature ([$GZMA + GZMB + PRF1 + INFG$]/4) was plotted as a function of the GMGS or GLS for basal-like breast cancer samples that are "glutamine metabolism high" (black) and "mixed metabolism" (pink). Linear regression lines of best fit are shown for both groups. Pearson's correlation analyses are shown. (**D**) Kaplan-Meier analysis of overall survival of basal breast tumors stratified based on the GMGS and the CTL gene signature: GMGS[LOW]/CTL[HIGH] (green, $n = 64$), GMGS[LOW]/CTL[LOW] (blue, $n = 14$), GMGS[HIGH]/CTL[HIGH] (orange, $n = 121$), and GMGS[HIGH]/CTL[LOW] (red, $n = 42$). Log-rank (Mantel-Cox) test: P values are shown. Red box indicates statistical significance after Bonferroni's correction for multiple comparisons. Hazard ratio (HR) (log-rank) and 95% confidence intervals are shown.

changes in tumor cell proliferation do not account for diminished tumor growth in the absence of GLS. Further, these data suggest that while E0771 murine TNBC tumor cells import and convert glutamine to glutamate, these tumors are not specifically dependent on glutamine metabolism for their growth in vivo, making these tumors an ideal model for assessing the impact of tumor glutamine metabolism on cells of the tumor microenvironment.

To evaluate the role of tumor-associated lymphocytes in the observed reduction of E0771-GLS[KO] tumor growth and metastasis, E0771-WT and E0771-GLS[KO] cells were inoculated into the mammary fat pads of Rag1 WT (*Rag1^{+/+}*) or Rag1-deficient (*Rag1^{-/-}*) mice, which lack mature B and T lymphocytes (35). In *Rag1^{+/+}* mice, E0771-GLS[KO] tumors grew more slowly than E0771-WT (Figure 2, E and F). However, E0771-GLS[KO] tumors grew at a similar rate to E0771-WT tumors when grown in the lymphocyte-

devoid microenvironment of *Rag1^{-/-}* mice. Further, IHC for the apoptosis marker cleaved caspase-3 was elevated in E0771-GLS[KO] tumors grown in lymphocyte-replete *Rag1^{+/+}* mice, but not in lymphocyte-devoid *Rag1^{-/-}* mice (Figure 2G). Similarly, fewer metastatic lung lesions were observed in E0771-GLS[KO] tumor-bearing *Rag1^{+/+}* mice, but this difference was lost in *Rag1^{-/-}* animals (Figure 2H and I). Together, these data suggest that lymphocytes are necessary to induce tumor cell death and reduce tumor metastasis in E0771-GLS[KO] tumors.

Loss of GLS in mammary epithelia reduces tumor latency and growth, while significantly enhancing T cell activation in the C3(1)-TAg transgenic mouse model. To assess the role of TNBC cell glutamine metabolism on multistep tumorigenesis, we used a genetically engineered spontaneous model, *C3(1)-TAg*, which expresses the SV40 large T antigen (TAg) in mammary epithelial cells of female
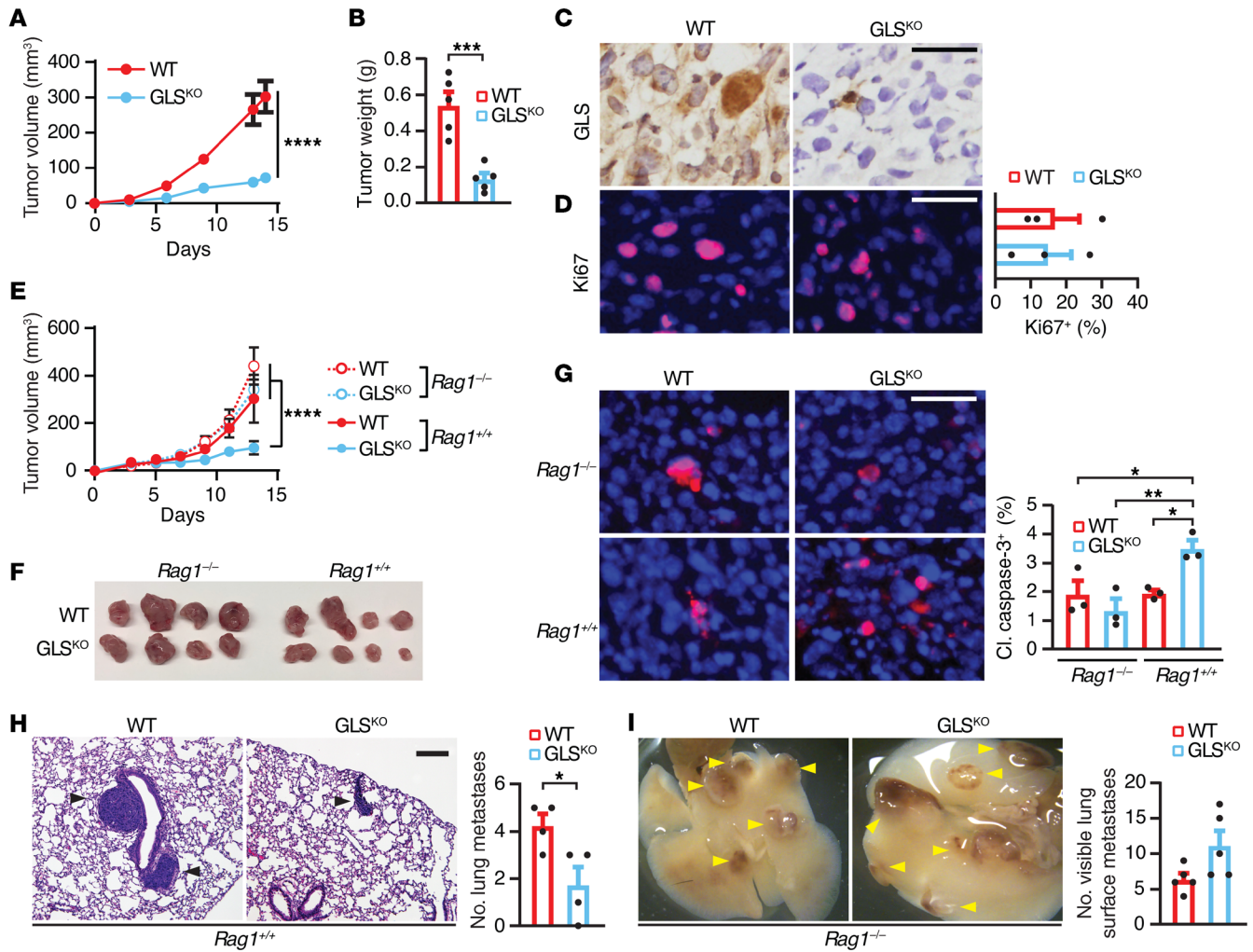
Figure 2. Loss of tumor cell–specific GLS reduces tumor growth and metastasis in a lymphocyte-dependent manner in an orthotopic model of TNBC. (**A–D**) E0771 sgLacZ (E0771-WT, red) or sgGls_3C15 (E0771-GLS^{KO}, blue) (5×10^5) cells were implanted into the number 4 mammary fat pad of female C57BL/6 mice (Jackson Laboratories), and tumors were harvested after 14 days. (**A**) Tumor volume ($P = 1.19 \times 10^{-10}$ by 2-way ANOVA) and (**B**) tumor weight at harvest ($P = 7.94 \times 10^{-4}$ by unpaired Student's t test) are shown. $n = 5$ mice per group. (**C** and **D**) Tumor sections from **A** and **B** were stained by (**C**) immunohistochemistry for GLS (brown) or immunofluorescence for (**D**) Ki67 (red). Nuclei were stained with (**C**) hematoxylin (blue) or (**D**) DAPI (blue). Total nuclei and Ki67$^+$ cells were counted from 3 fields of view using ImageJ software. Scale bars: 10 μm (GLS) and 20 μm (Ki67). $P = 0.84$ by unpaired Student's t test. $n = 3$ mice per group. (**E–G**) E0771-WT or E0771-GLS^{KO} cells were implanted as described above in wild-type C57BL/6 ($Rag1^{+/+}$) or Rag1-deficient ($Rag1^{-/-}$) mice. (**E**) Plot of tumor volumes is shown. $P = 2.66 \times 10^{-6}$ by 2-way ANOVA. (**F**) Image of tumors harvested from **E** ($n = 4$ mice per group). (**G**) Immunofluorescence of cleaved caspase-3 (red). Nuclei were stained with DAPI (blue). $P = 0.011$ by 1-way ANOVA with Tukey's post hoc analysis for multiple comparisons. $n = 3$ mice per group. (**H** and **I**) C57BL/6 ($Rag1^{+/+}$) (**H**) or $Rag1^{-/-}$ (**I**) mice were implanted with E0771-WT or E0771-GLS^{KO} cells (2.5×10^5). (**H**) Lungs were harvested when tumors reached 400–600 mm³ (21–27 days). Micrometastases (black arrows) were scored from 3 H&E-stained sections isolated 100 μm apart ($n = 4$ mice per group). Scale bar: 200 μm. (**I**) Tumors were resected at 1400–1500 mm³, and lungs were harvested between 13 and 20 days after resection ($n = 5$ mice per group). Surface metastases (yellow arrows) were scored from whole lung specimens. Unpaired Student's t test: (**H**) $P = 0.031$, (**I**) $P = 0.062$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****$P < 0.001$.

mice via the promoter for the C3(1) component of the rat prostate steroid binding protein (PSBP) (36). Importantly, genetic profiling studies showed that the *C3(1)-TAg* mouse model recapitulated BLBC gene signatures seen in human TNBC/BLBC specimens, including an abundance of genes representing proliferation, cell cycle pathways, and chromosome instability gene signatures (37, 38). *C3(1)-TAg* mice were crossed into genetically engineered mice with floxed *GLS* alleles (*GLS*$^{fl/fl}$) (26) and with transgenic *MMTV-Cre* mice expressing Cre recombinase (39), allowing for Cre-mediated recombination at floxed *GLS* alleles in mammary epi-

thelial cells (39). While *C3(1)-TAg; GLS*$^{fl/fl}$ mice lacking *MMTV-Cre* (referred to here as *TAg-GLS*$^{fl/fl}$) developed tumors with an average latency of 34.4 weeks, latency was significantly delayed to 40.2 weeks in *C3(1)-TAg; MMTV-Cre; GLS*$^{fl/fl}$ (referred to as *TAg-GLS*$^{Δ/Δ}$) (Figure 3A). Kaplan-Meier analysis of survival in tumor-bearing mice revealed extended survival in *TAg-GLS*$^{Δ/Δ}$ mice as compared with *TAg-GLS*$^{fl/fl}$ (Figure 3B). To rule out the potentially skewing effects of delayed tumor latency on apparent tumor volume measurements as a function of mouse age, we instead set the time of initial tumor palpation as $t = 0$, and then assessed tumor volumes
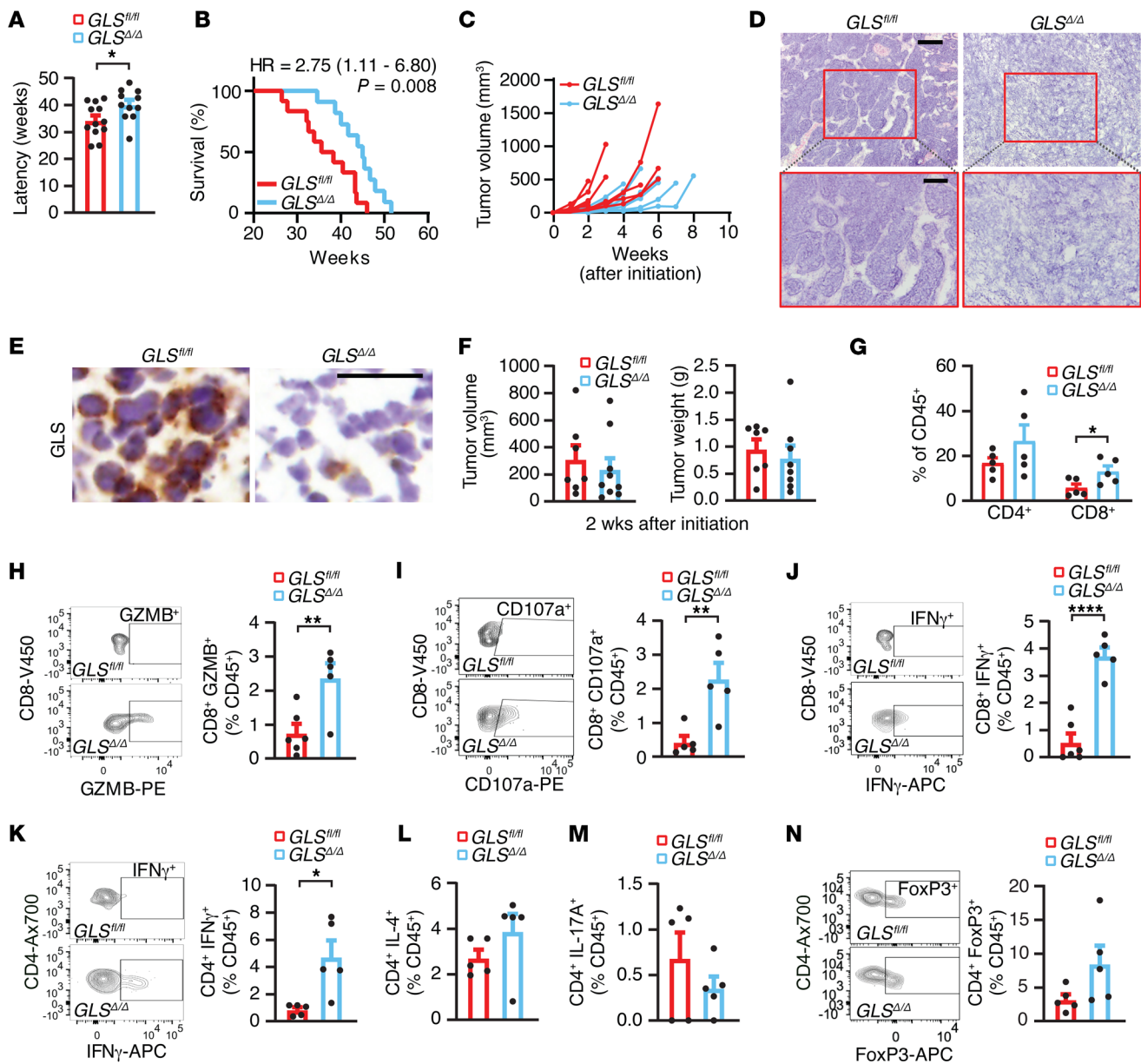
Figure 3. Mammary-specific loss of GLS in a spontaneous TNBC tumor model delays tumor initiation and improves activation of T cells. *C3(1)-TAg/GLS^{fl/fl}* (*GLS^{fl/fl}*, red) or *C3(1)-TAg; MMTV-Cre; GLS^{fl/fl}* (*GLS^{Δ/Δ}*, blue) mice were palpated weekly for tumor formation and progression. (**A**) Tumor latency for *GLS^{fl/fl}* or *GLS^{Δ/Δ}* mice was recorded as the age (weeks) of initial tumor detection. *P* = 0.031 by unpaired Student's *t* test. *n* = 11–12 mice per group. (**B**) Survival (weeks) was determined by the humane endpoint for the *GLS^{fl/fl}* or *GLS^{Δ/Δ}* from **A**, plotted as the percentage of surviving mice as a function of age (weeks). *P* = 0.0082 by Gehan-Breslow-Wilcoxon test. Hazard ratio was calculated using log-rank analysis, with the 95% confidence interval shown. (**C**) Plot of tumor volume after tumor initiation in mice described in **A**. (**D**) H&E images of *GLS^{fl/fl}* or *GLS^{Δ/Δ}* tumors. Scale bars: 200 μm (top) and 100 μm (bottom). (**E**) Immunohistochemistry for GLS (brown) of *GLS^{fl/fl}* or *GLS^{Δ/Δ}* tumors. Nuclei were stained with hematoxylin (blue). Scale bar: 20 μm. (**F**) Tumors were harvested from *GLS^{fl/fl}* (red) or *GLS^{Δ/Δ}* (blue) mice at 1 to 2 weeks after initial tumor detection. Tumor volume (mm³) (left) and tumor mass (grams) (right) were recorded at harvest. Unpaired Student's *t* test: *P* = 0.581 (volume), *P* = 0.581 (mass). *n* = 7–9 mice per group. (**G–N**) Flow cytometric analyses of whole tumor preparations. *n* = 5–6 mice per group. (**G**) CD4+ (left) or CD8+ (right) T cells, plotted as percentage of CD45+ immune cells. Unpaired Student's *t* test: *P* = 0.226 (CD4+), *P* = 0.043 (CD8+). (**H–N**) Flow cytometric analyses of (**H**) CD8+GZMB+, (**I**) CD8+CD107a+, (**J**) CD8+IFN-γ+, (**K**) CD4+IFN-γ+, (**L**) CD4+IL-4+, (**M**) CD4+IL-17A+, and (**N**) CD4+FoxP3+ T cells in GLS^{fl/fl} (red) or *GLS^{Δ/Δ}* (blue) tumors, plotted as a percentage of CD45+ cells. Unpaired Student's *t* test: *P* = 0.0085 (CD8+GZMB+), *P* = 0.0056 (CD8+CD107a+), *P* = 4.94 × 10^{-5} (CD8+IFN-γ+), *P* = 0.011 (CD4+IFN-γ+), *P* = 0.212 (CD4+IL-4+), *P* = 0.322 (CD4+IL-17A+), *P* = 0.093 (CD4+ FoxP3+). **P* < 0.05, ***P* < 0.01, *****P* < 0.001.

in the weeks following initial tumor palpation. This approach revealed markedly reduced growth of *TAg-GLS^{Δ/Δ}* tumors as compared with *TAg-GLS^{fl/fl}* (Figure 3C). Histological analysis of tumors collected from mice at humane endpoints revealed dense, high-

ly cellular tumors with tightly packed nuclei in *TAg-GLS^{fl/fl}* mice. However, vast central areas lacking tumor cellularity were noted in *TAg-GLS^{Δ/Δ}* tumors (Figure 3D). IHC detection of GLS confirmed loss of GLS protein expression in *TAg-GLS^{Δ/Δ}* tumors (Figure 3E).

In order to assess how tumor cell–specific GLS impacts T cells within the tumor microenvironment, T cell populations were analyzed from tumor single-cell suspensions using flow cytometry (Supplemental Figure 3A). Because tumor size may affect antitumor immunity, we analyzed tumors harvested at 2 weeks after initial tumor palpation, a time point when both groups displayed similar tumor volumes and weights (Figure 3F). *TAg-GLS*$^{\Delta/\Delta}$ tumors harbored significantly increased levels of CD45$^+$ immune cells and CD45$^+$CD3$^+$ TILs (Supplemental Figure 3, B and C), with greater enrichment of CD3$^+$CD8$^+$ T cells (Figure 3G and Supplemental Figure 3D). Cytotoxic CD8$^+$ T cells directly carry out their killing activity via release of the serine protease, granzyme B (GZMB) (40), and consistent with increased effector function, *TAg-GLS*$^{\Delta/\Delta}$ tumors harbored increased GZMB$^+$CD8$^+$ T cells compared with *TAg-GLS*$^{fl/fl}$ controls (Figure 3H and Supplemental Figure 3E). *TAg-GLS*$^{\Delta/\Delta}$ tumors also displayed increased cytotoxic T lymphocytes (CTLs) expressing the degranulation marker CD107a (Figure 3I and Supplemental Figure 3F), the proinflammatory cytokine interferon γ (IFN-γ) (Figure 3J, and Supplemental Figure 3G), as well as the cell activation markers CD25 and CD44 (Supplemental Figure 3, H–J). Tumor cell loss of GLS also resulted in a modest increase in both the number of IFN-γ–producing CD4$^+$ Th1 T helper cells and the IFN-γ median fluorescence intensity (Figure 3K and Supplemental Figure 3K). Smaller increases were observed in the IL-4$^+$ Th2 as well as the FoxP3$^+$ and FoxP3$^+$CD25$^+$ Treg CD4$^+$ T cell populations in *TAg-GLS*$^{\Delta/\Delta}$ tumors, whereas a small decrease in the IL-17A$^+$ Th17 population was detected, although these changes were not associated with increased cytokine production or FoxP3 expression (Figure 3, L–N, and Supplemental Figure 3, L–Q). Interestingly, the proportion of CD4$^+$ T cells expressing activation markers CD25, CD69, and CD44 was largely unchanged, suggesting that cumulative CD4$^+$ T cell activation may not be occurring in *TAg-GLS*$^{\Delta/\Delta}$ tumors (Supplemental Figure 3, R–T). Together, these findings suggest that targeting tumor-specific GLS improves CD8$^+$ T cell activation and effector capacity with this TNBC model.

Tumor cell GLS deficiency in established tumors increases T cell numbers and effector function. T cell numbers and activation markers were also examined in orthotopic E0771-WT and E0771-GLS^{KO} tumors grown in WT C57BL/6 mice. Flow cytometric tumor analyses identified increased numbers of CD45$^+$ tumor-associated leukocytes and CD45$^+$CD3$^+$ T cells in E0771-GLS^{KO} tumors as compared with E0771-WT (Supplemental Figure 4, A and B). The percentage of the CD45$^+$ population that was also CD3$^+$CD8$^+$ was increased in E0771-GLS^{KO} tumors compared with the WT controls (Figure 4A). A trend toward an increased percentage of CD45$^+$ tumor leukocytes that were CD4$^+$ was also seen in E0771-GLS^{KO} tumors. When normalized to total tumor mass, the numbers of both CD4$^+$ and CD8$^+$ T cells were increased in E0771-GLS^{KO} tumors as compared with controls (Figure 4B).

Further, CD8$^+$ T cells expressing mature CTL effector markers were increased in E0771-GLS^{KO} tumors, including those expressing GZMB (Figure 4C), CD107a (Figure 4D), and IFN-γ (Figure 4E), while CTL activation markers were also increased, including CD25, CD69, and CD44, (Supplemental Figure 4, C–E), suggesting that GLS loss from the tumor epithelia may indirectly enhance the differentiation and effector activity of CD8$^+$ T cells. E0771-GLS^{KO} tumors also exhibited an increased presence of CD4$^+$IFN-γ$^+$ Th1

cells (Figure 4F), although the number of the CD4$^+$IL-4$^+$ Th2 cells was not significantly altered by GLS loss from tumor cells (Figure 4G). Interestingly, CD4$^+$IL-17A$^+$ Th17 populations were also increased in E0771-GLS^{KO} tumors (Figure 4H and Supplemental Figure 4F), although no significant changes were observed in Tregs (Figure 4I and Supplemental Figure 4F). Additionally, the number of CD4$^+$ T cells from E0771-GLS^{KO} tumors expressing the activation markers CD25, CD69, and CD44 was increased, consistent with increased CD4$^+$ T cell activation (Supplemental Figure 4, G–I). Collectively, these data suggest that deletion of GLS in TNBC tumor cells enhances the number and effector function of tumor-infiltrating T cells, possibly through changes in the metabolite availability within the tumor microenvironment in which these lymphocytes must function.

Loss of GLS in tumor cells increases interstitial glutamine and enhances antigen-specific antitumor CD8$^+$ T cell infiltration. Glutamine is an abundant amino acid in the blood stream, but glutamine supply can vary in the tissue interstitium (41, 42). Notably, glutamine concentrations measured within tumor interstitial fluid collected from E0771-WT tumors was approximately 100 μM, whereas E0771-GLS^{KO} interstitial tumor glutamine levels were nearly 700 μM (Figure 4J), similar to the reported plasma glutamine concentration of C57BL/6 mice (41, 43). These data suggest that GLS loss from tumor cells prevents tumor cell–driven glutamine depletion within the interstitium, which may provide more available glutamine for other cells in the tumor microenvironment, including lymphocytes. Indeed, we observe that IFN-γ secretion from effector type I CD8$^+$ T cells (Tc1) was significantly increased in response to elevated glutamine (Figure 4K), suggesting that greater glutamine availability in GLS^{KO} tumor interstitium may directly improve CTL effector responses.

To analyze how tumor cell glutamine metabolism may regulate an antigen-specific T cell response, we utilized E0771(OVA)-WT and E0771(OVA)-GLS^{KO} cells expressing the full-length ovalbumin (OVA) protein (Supplemental Figure 4, J and K). OVA peptides are processed for presentation of the OVA$_{257–264}$ antigenic peptide (SIINFEKL) on MHC class I (H2K^b) molecules (44). CD8$^+$ T cells expressing a SIINFEKL-specific T cell receptor (TCR) (45, 46) were purified from OT-I transgenic mice (Supplemental Figure 4L), transduced ex vivo to overexpress GFP, and then primed with SIINFEKL peptide. Following E0771(OVA) tumor engraftment, adoptive transfer of OT-I GFP$^+$CD8$^+$ T cells into tumor-bearing mice enabled immunofluorescent visualization of CTL tumor infiltration (Figure 4L). Although OT-I GFP$^+$CD8$^+$ T cells were detected in all tumors, GFP$^+$ cells were more abundant in E0771(OVA)-GLS^{KO} tumors (Figure 4M). Flow cytometry confirmed this observation, revealing nearly twice as many GFP$^+$CD8$^+$ T cells from E0771-GLS^{KO} tumors as compared with E0771-WT tumors (Figure 4N). Together, these results suggest that inhibition of glutamine utilization within tumor cells increases the intratumoral CD8$^+$ T cells.

Glutamine transporter inhibition by V-9302 reduces tumor growth and improves T cell activation. Because genetic models of impaired tumor cell glutaminolysis resulted in decreased tumor growth, increased glutamine concentrations in the tumor interstitium, and improved TIL numbers, we tested the hypothesis that pharmacologic blockade of glutamine metabolism might significantly
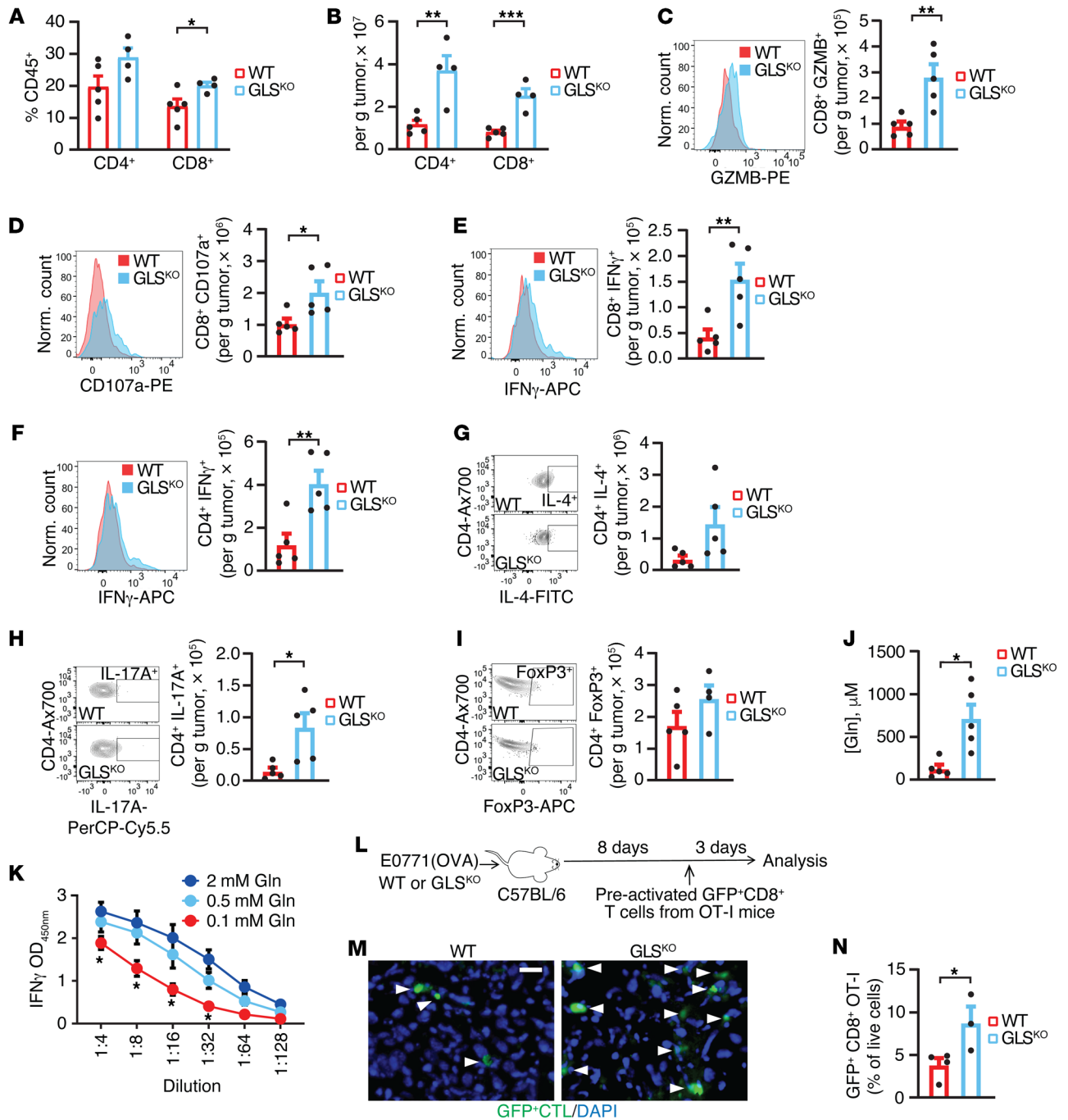
Figure 4. Loss of tumor cell–specific GLS increases T cell activation in an orthotopic TNBC tumor model. (**A**–**I**) E0771 sgLacZ (WT, red) or sgGls_3C15 (GLS^KO, blue) (5 × 10^5) cells were implanted into the number 4 mammary fat pad of female C57BL/6 mice (Jackson Laboratories). Tumors were harvested after 14 days. n = 4–5 mice per group. (**A** and **B**) Flow cytometric analyses of CD4^+ (left) or CD8^+ (right) T cells, plotted as percentage of (**A**) CD45^+ immune cells or (**B**) total cells normalized to tumor mass (gram). Unpaired Student's t test: CD4^+, (**A**) P = 0.091, (**B**) P = 0.0063; CD8^+, (**A**) P = 0.040, (**B**) P = 8.23 × 10^{-4}. (**C**–**I**) Flow cytometric analyses of tumors for (**C**) CD8^+GZMB^+, (**D**) CD8^+CD107a^+, (**E**) CD8^+IFN-γ^+, (**F**) CD4^+IFN-γ^+, (**G**) CD4^+IL-4^+, (**H**) CD4^+IL-17A^+, and (**I**) CD4^+FoxP3^+ CD45^+CD3^+ T cells. Total counts were normalized per tumor mass (grams). Unpaired Student's t test: P = 0.0064 (CD8^+GZMB^+), P = 0.027 (CD8^+CD107a^+), P = 0.0091 (CD8^+IFN-γ^+), P = 0.0065 (CD4^+IFN-γ^+), P = 0.065 (CD4^+IL-4^+), P = 0.017 (CD4^+IL-17A^+), P = 0.198 (CD4^+FoxP3^+). (**J**) Tumor interstitial fluid was collected from E0771-WT (red) or E0771-GLS^KO (blue) tumors via centrifugation of harvested tumors. Glutamine (gln) was measured and concentration (μM) was calculated against a glutamine standard. P = 0.021 by paired Student's t test. n = 5 mice per group. (**K**) IFN-γ ELISA of supernatants collected from Tc1 CD8^+ T cells activated (anti-CD3/anti-CD28) in media supplemented with 0.1, 0.5, or 2 mM glutamine (n = 3). Two-way ANOVA; unpaired Student's t test. (**L**–**N**) E0771(OVA) sgLacZ (WT, red) or sgGls3 (GLS^KO, blue) (5 × 10^5) cells were bilaterally implanted into the number 4 mammary fat pads of female C57BL/6 mice. (**L**) Experimental timeline is shown. (**M**) Immunofluorescence of GFP^+CD8^+ OT-I (GFP^+CTLs) cells in tumors. Nuclei were stained with DAPI (blue). Arrows indicate GFP^+CD8^+ OT-I T cells. Scale bar: 20 μm. (**N**) Flow cytometric analysis of GFP^+CD8^+ T cells from whole cell tumor preparations, plotted as percentage of all live cells. P = 0.043 by unpaired Student's t test. n = 3–4 mice per group. *P < 0.05, **P < 0.01, ***P < 0.005.

diminish growth and support T cell activity in TNBC models. Early clinical studies of the GLS inhibitor CB-839 demonstrated tolerability and safety, but with limited efficacy as a single agent in TNBC (47). However, V-9302, a small molecule amino acid transporter inhibitor with a greater selectivity for reducing glutamine uptake (48, 49), was more efficacious for inducing TNBC cell death in cell culture models, including the human TNBC lines MDA-MB-231 and HCC1806 and the mouse TNBC lines 4T1 and E0771 (Supplemental Figure 5, A–D). Furthermore, V-9302 reportedly has little to no negative impact on CD44 expression on CD8+ T cells ex vivo (48). To test the impact of glutamine uptake inhibition with V-9302, orthotopic E0771 tumors grown in immune-competent C57BL/6 female mice were treated daily with V-9302 (50 mg/kg) or vehicle beginning when tumors reached 100 mm³, equivalent to 11 days after tumor cell inoculation. Tumors treated with V-9302 displayed markedly reduced tumor growth (Figure 5A), resulting in decreased tumor weight upon collection on day 16, after only 5 days of treatment (Figure 5B). While V-9302 had only a marginal impact on Ki67+ cell proliferation, there was a more significant (3-fold) increase in apoptosis, as measured by cleaved caspase-3 (Figure 5C), in agreement with the increased cell death seen upon genetic GLS loss in E0771 tumors.

Interestingly, the number of CD45+ leukocytes was not significantly impacted by 5 days of V-9302 treatment (Supplemental Figure 5E), nor were the total number of CD45+CD3+ T cells (Supplemental Figure 5F). While total CD8+ T cell frequencies were unaffected by V-9302 treatment (Figure 5D), detailed histological assessment identified CD8+ T cells as being restricted to the periphery of vehicle-treated E0771 tumors, while an increased CD8+ T cell infiltration deeper into the tumor core (>500 μm) was seen in tumors treated with V-9302 (Figure 5E). Changes in localization from the periphery to the intratumoral core have been described previously, correlating with immune response to therapeutic immune checkpoint inhibition in several tumor models (50–52). We next assessed how V-9302 impacted CD8+ TIL activation and effector markers. Tumors treated with V-9302 harbored CD8+ T cell populations producing GZMB (Figure 5F), CD107a (Figure 5G), IFN-γ (Figure 5H), CD25, CD69, and CD44 (Supplemental Figure 5, G and H), confirming CTL effector capacity and activation in response to V-9302. Additionally, CD4+ TILs from tumors treated with V-9302 displayed an increased population of IFN-γ–producing cells, suggesting an increased CD4+ Th1 T cell phenotype upon V-9302 treatment (Figure 5I). Only smaller increases in the Th2 (IL-4) and Th17 (IL-17A) populations were observed (Figure 5J and Supplemental Figure 5I). In contrast, the CD4+FoxP3+CD25+CD127lo Treg population was significantly decreased in V-9302–treated tumors, although the FoxP3+ population was not changed (Figure 5K and Supplemental Figure 5J). The frequency of CD4+ T cell populations expressing the activation markers CD25, CD69, and CD44 were also increased (Supplemental Figure 5, K and L). Together, these data confirm that V-9302 increases tumor cell death while simultaneously augmenting CD8+ CTLs and CD4+ Th1 phenotypes within the TNBC tumor microenvironment.

Compared with effector memory (Tem) or terminally differentiated effector T cells, CD8+ central memory T cells (Tcm) support a more persistent antitumor response (53, 54). To assess whether V-9302 may support long-term cytotoxicity, we evaluated the CD44+CD62L+ Tcm and CD44+CD62L− Tem populations. Treatment of ex vivo–activated splenocytes with V-9302 significantly increased the Tcm/Tem ratio of CD8+ T cells but not CD4+ T cells (Supplemental Figure 5, M and N). However, both the Tcm and Tem populations were increased in V-9302–treated tumors (Supplemental Figure 5, O and P), suggesting that V-9302 improves antitumor responses in part through supporting persistent memory T cells.

The glutamine transporter inhibitor V-9302 increases glutamine uptake and glutathione synthesis in CTLs. To investigate the underlying molecular mechanisms regulating increased T cell activation upon treatment with V-9302, splenocytes harvested from WT mice and cultured ex vivo were primed with anti-CD3 and anti-CD28 antibodies in the presence or absence of V-9302 (10 μM for 48 hours). As expected, anti-CD3/anti-CD28 priming increased the viable percentage of the total CD8+ T cell population, as compared with splenocytes not primed with anti-CD3/anti-CD28 (Supplemental Figure 6A). Importantly, V-9302 did not affect the proportion of viable CD8+ T cells following anti-CD3/anti-CD28 priming (Supplemental Figure 6A), nor did V-9302 affect the viable CD4+ T cell population (Supplemental Figure 6B). Previous studies demonstrate that anti-CD3/anti-CD28 priming of T cells activates the mTORC1 signaling pathway, resulting in phosphorylation of the mTORC1 effector ribosomal protein S6 (S6RP) (55, 56). As measured by phos-flow cytometry, anti-CD3/anti-CD28 priming of CD8+ T cells increased phospho-S6RP, which was unaffected by V-9302 (Supplemental Figure 6C). Similarly, V-9302 did not significantly alter phospho-S6RP in CD4+ T cells (Supplemental Figure 6D). The cytotoxic capabilities of OT-I CD8+ T cells against E0771(OVA) tumor cells were assessed in coculture studies performed in the presence and absence of V-9302. Importantly, OT-I CD8+ T cells were poorly cytotoxic against parental E0771 cells, but displayed potent cytotoxicity against E0771(OVA) cells, confirming antigen-directed cytotoxicity against tumors cells (Figure 6A). Notably, treatment with V-9302 increased T cell–dependent cytotoxicity in this assay nearly 2-fold, indicating that V-9302 enhances the ability of CD8+ T lymphocytes to kill tumor cells. These studies suggest that V-9302 enhances the cytotoxicity of CD8+ T cells but has no significant impact on mTORC1 activation or T cell viability upon TCR stimulation, despite the well-documented requirement for glutamine uptake and glutaminolysis in T cell viability and activation.

Still, similar to our observation in a GLS deletion model, V-9302 increased the tumor interstitial glutamine concentration (Figure 6B), indicating that V-9302 may improve glutamine availability for T cells. These observations were assessed further by measuring ³H-glutamine uptake in anti-CD3/anti-CD28–primed CD8+ T cells cocultured with E0771 tumor cells. After pulsing for 15 minutes with ³H-glutamine, tumor cells and CD8+ T cells were assessed separately for ³H-glutamine uptake. While ³H-glutamine was detected in E0771 cells upon coculture with CD8+ T cells, treatment of cocultures with V-9302 diminished ³H-glutamine uptake by E0771 tumors nearly 40% (Figure 6C). In contrast, V-9302 treatment did not diminish ³H-glutamine uptake by CD8+ T cells, and even increased CD8+ T cell ³H-glutamine uptake, a differential effect that was also seen in isolated cells (Supplemen-
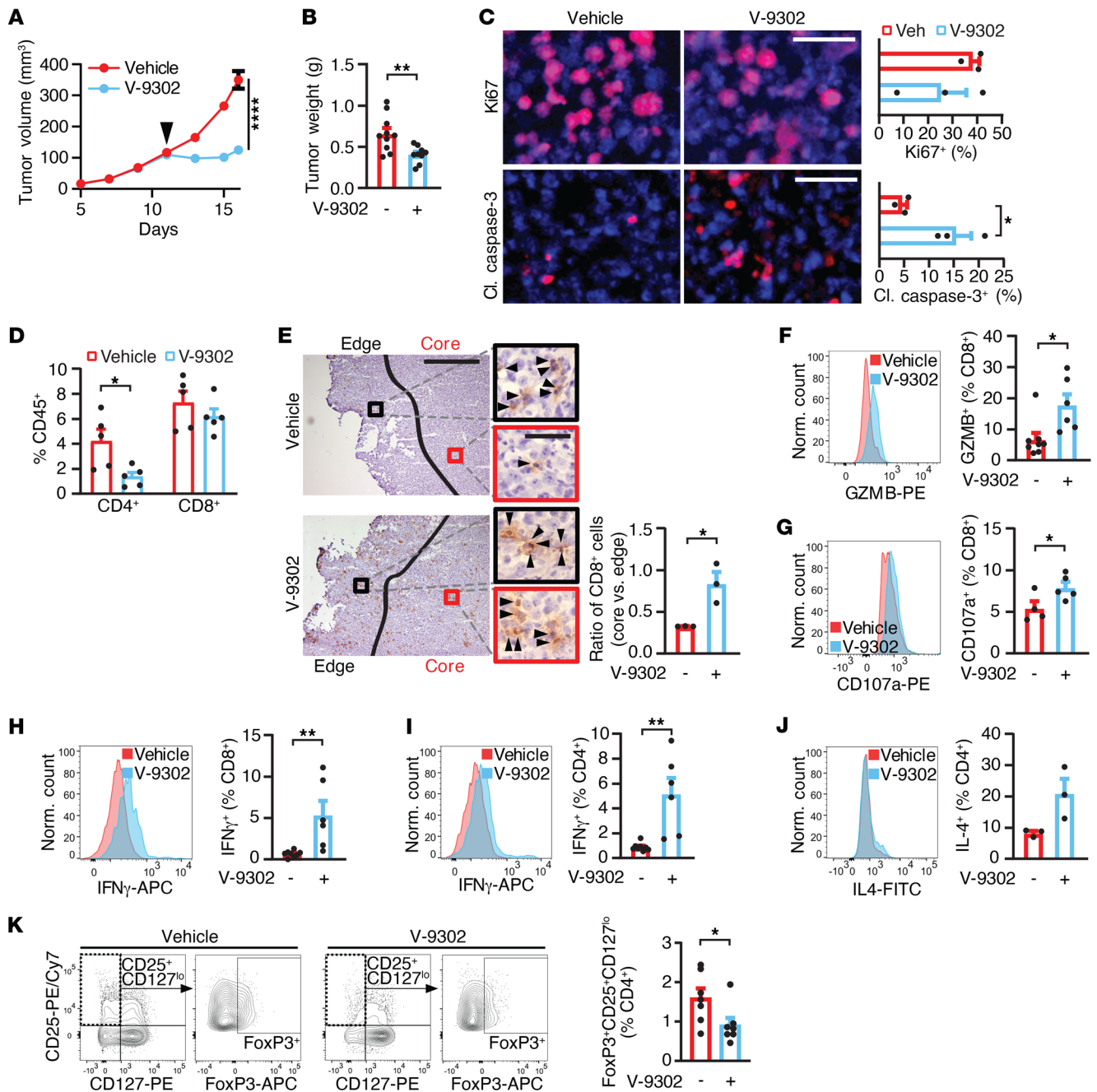
Figure 5. The glutamine transporter inhibitor V-9302 suppresses tumor growth and increases T lymphocyte activation in a model of TNBC. E0771 cells (2.5×10^5) were bilaterally injected into the number 4 mammary fat pads of female C57BL/6 mice (Taconic). Beginning on day 11, mice were treated with vehicle (DMSO, red) or 50 mg/kg V-9302 (blue) daily for 5 days. (**A**) Tumor volume was measured over time. Arrow indicates beginning of treatment. $P < 1 \times 10^{-15}$ by 2-way ANOVA. n = 9–10 mice per group. (**B**) Average tumor mass per mouse at harvest. P = 0.0060 by unpaired Student's t test. n = 9–10 mice per group. (**C**) Immunofluorescence of tumor sections for Ki67 (top) or cleaved caspase-3 (bottom), both red. Nuclei were stained with DAPI (blue). Scale bars: 20 μm. Ki67+, cleaved caspase-3+, and nuclei were averaged from 3 fields of view. Unpaired Student's t test: P = 0.283 (Ki67), P = 0.023 (cleaved caspase-3). (**D**) Flow cytometric analyses of CD4+ (left) or CD8+ (right) T cells, plotted as a percentage of CD45+ immune cells, in vehicle- (red) or V-9302–treated (blue) tumors. Unpaired Student's t test: P = 0.023 (CD4+), P = 0.340 (CD8+). n = 5 mice per group. (**E**) Immunohistochemistry of CD8a (brown) from vehicle- or V-9302–treated (50 mg/kg) tumors. Nuclei were stained with hematoxylin (blue). Edge (black) is considered <500 μm (denoted by solid line) from tumor margin, core (red) is >500 μm. Scale bars: 500 μm and 50 μm for expanded and enlarged images, respectively. CD8+ cells (denoted by arrows) were averaged from 3 fields of view and normalized to field-of-view area. P = 0.019 by unpaired Student's t test. n = 3 mice per group. (**F–K**) Flow cytometric analyses of (**F**) CD8+GZMB+, (**G**) CD8+CD107a+, (**H**) CD8+IFN-γ+, (**I**) CD4+IFN-γ+, (**J**) CD4+IL-4+, and (**K**) CD4+FoxP3+CD25+CD127loCD45+CD3+ T cells in vehicle- (red) or V-9302–treated (blue) tumors. Unpaired Student's t test: P = 0.014 (CD8+GZMB+), P = 0.047 (CD8+CD107a+), P = 0.007 (CD8+IFN-γ+), P = 0.0021 (CD4+IFN-γ+), P = 0.057 (CD4+IL-4+), P = 0.034 (CD4+FoxP3+CD25+CD127lo). n = 3–8 mice per group. *P < 0.05, **P < 0.01, ****P < 0.001.
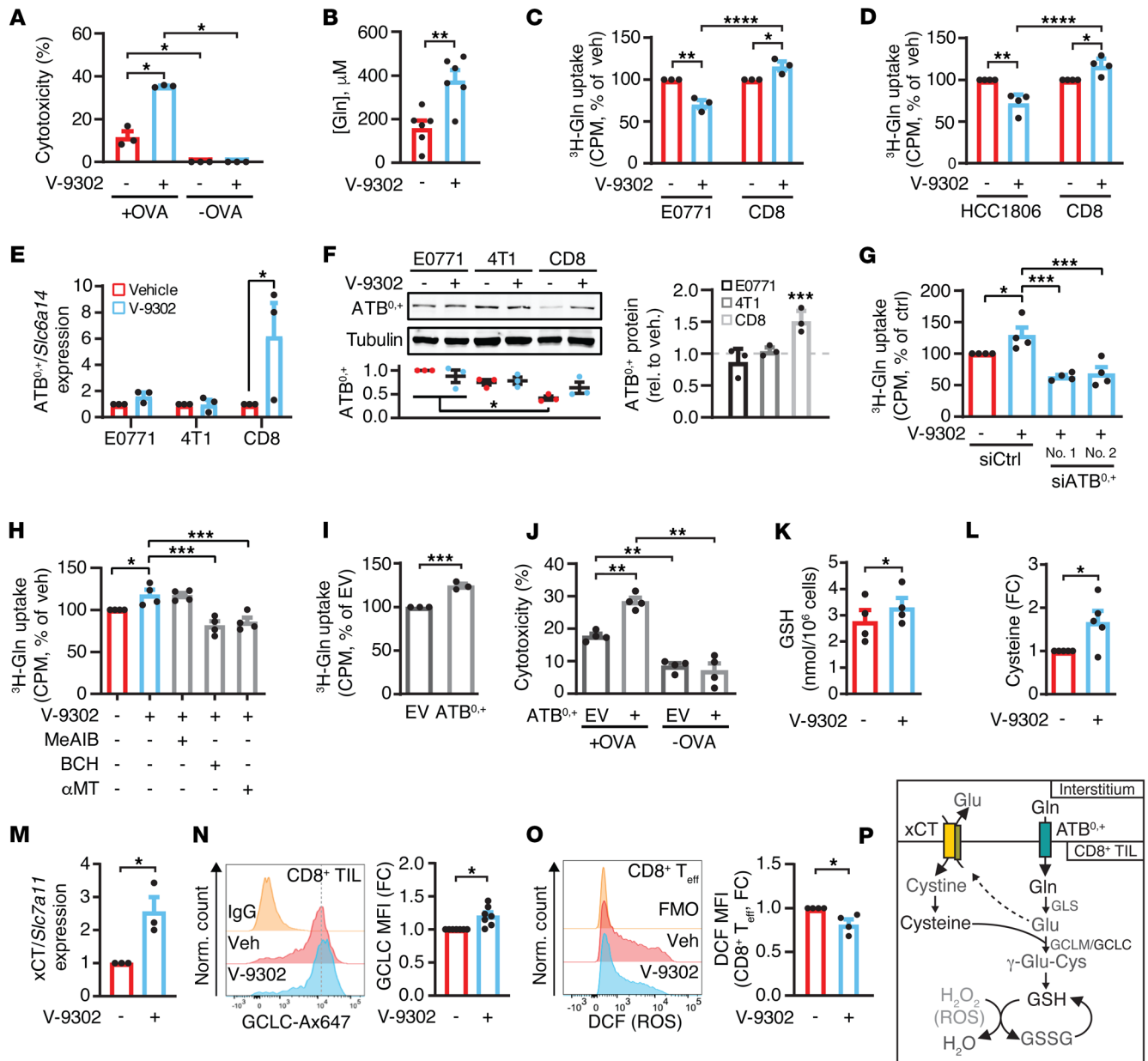
Figure 6. V-9302 induces ATB⁰,⁺ expression to sustain glutamine uptake and glutathione synthesis in activated CD8⁺ T cells. (**A**) LDH cytotoxicity assay of E0771(OVA) and CD8⁺ OT-I CTLs cocultured in vehicle (red) or V-9302 (blue) ($n = 3$). $P = 0.0018$ by 1-way ANOVA with Tukey's post hoc test. (**B**) Tumor interstitial fluid glutamine in vehicle- or V-9302–treated tumors. $P = 0.0027$ by unpaired Student's t test. $n = 6$ mice per group. (**C** and **D**) ³H-glutamine uptake assay in (**C**) E0771 and C567BL/6 CD8⁺ CTLs or (**D**) HCC1806 and human CD8⁺ CTLs isolated from PBMCs in vehicle or V-9302 ($n = 3–4$). Averaged triplicate radioactivity (CPM) was normalized to vehicle. Two-way ANOVA (E0771: $P = 2.18 \times 10^{-4}$; HCC1806: $P = 1.43 \times 10^{-4}$) with Tukey's post hoc test. (**E**) Relative expression of ATB⁰,⁺/*Slc6a14* ($n = 3$). $P = 0.036$ by 2-way ANOVA with Tukey's post hoc test. (**F**) ATB⁰,⁺ Western blot of cells from **E**. Relative ATB⁰,⁺ protein was normalized as indicated ($n = 3$). Two-way ANOVA (bottom, $P = 0.0014$; right, $P = 0.0026$) with Tukey's (bottom) or Sidak's (right) post hoc test. (**G** and **H**) ³H-glutamine uptake of CD8⁺ CTLs (**G**) after *ATB⁰,⁺* knockdown or (**H**) in the presence of glutamine transporter inhibitors, as indicated ($n = 4$). One-way ANOVA: $P = 4.98 \times 10^{-5}$ (**G**), $P = 3.41 \times 10^{-4}$ (**H**); Holm-Sidak post hoc test. (**I** and **J**) CD8⁺ CTLs overexpressing ATB⁰,⁺ were evaluated for (**I**) ³H-glutamine uptake or (**J**) LDH cytotoxicity ($n = 3–4$). Empty vector (EV) was used as a control. Unpaired Student's t test ($P = 7.39 \times 10^{-4}$) (**I**) or 1-way ANOVA ($P = 4.13 \times 10^{-5}$) with Tukey's post hoc test (**J**). (**K** and **L**) Intracellular (**K**) glutathione (GSH) and (**L**) cysteine in CD8⁺ CTLs ($n = 4–5$). Paired ($P = 0.020$) (**K**) or unpaired ($P = 0.027$) (**L**) Student's t test. (**M**) xCT/*Slc7a11* expression in activated CD8⁺ CTLs ($n = 3$). $P = 0.025$ by unpaired Student's t test. (**N**) GCLC protein expression in CD8⁺ TILs in tumors of vehicle- or V-9302–treated mice. Dotted line denotes vehicle peak in representative data shown. $P = 0.0034$ by unpaired Student's t test. $n = 7$ mice per group. (**O**) ROS in activated CD8⁺CD44⁺CD62L⁻ effector T cells determined by H₂-DCFDA ($n = 4$). $P = 0.016$ by unpaired Student's t test. (**P**) Proposed model of V-9302–mediated increases (bold) and decreases (gray) in CD8⁺ TILs. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$, $****P < 0.001$.

tal Figure 7, A and B). Similar results were seen when this experiment was repeated in HCC1806 human TNBC cells cocultured with human peripheral CD8+ lymphocytes (Figure 6D), confirming that V-9302 affected glutamine uptake by TNBC cells, but not by CD8+ T cells. Importantly, expression of ASCT2 (*Slc1a5*), the sodium-coupled neutral amino acid transporter family members SNAT1 (*Slc38a1*) and SNAT2 (*Slc38a2*), as well as the L-type amino acid transporter LAT1 (*Slc7a5*) and its cotransporter 4F2HC (*Slc3a2*), were unchanged in E0771, 4T1, and CD8+ T cells in the presence of V-9302 (Supplemental Figure 7, C–G). Interestingly, the Na+/Cl−–dependent neutral and cationic amino acid transporter ATB$^{0,+}$ (*Slc6a14*) was upregulated nearly 6-fold in CD8+ T cells, but not TNBC tumor cells, treated with V-9302 (Figure 6E). Despite low ATB$^{0,+}$ protein expression in control CD8+ T cells, Western blot analysis confirmed ATB$^{0,+}$ protein upregulation in CD8+ T cells in response to V-9302 treatment to levels more comparable to those observed in TNBC cells (Figure 6F).

To assess whether ATB$^{0,+}$/*Slc6a14* may compensate for glutamine transporter inhibition by V-9302 in CD8+ T cells, we evaluated ³H-glutamine uptake in anti-CD3/anti-CD28–primed CD8+ T cells following ATB$^{0,+}$ knockdown (Figure 6G and Supplemental Figure 7H). Consistent with our coculture system, V-9302 increases ³H-glutamine uptake in isolated CD8+ T cells (Figure 6G). However, ³H-glutamine uptake was significantly reduced following ATB$^{0,+}$ knockdown (Figure 6G). Likewise, inhibition of ATB$^{0,+}$ using 2-aminobicyclo(2.2.1)heptane-2-carboxylic acid (BCH) (57), an inhibitor of LAT1 and ATB$^{0,+}$, or α-methyl-DL-tryptophan (αMT), a selective ATB$^{0,+}$ inhibitor (58), significantly reduced ³H-glutamine uptake in anti-CD3/anti-CD28–primed CD8+ T cells (Figure 6H). In contrast, *N*-methyl-aminoisobutyric acid (MeAIB), an inhibitor of SNAT1/SNAT2 (49), had no effect on ³H-glutamine uptake (Figure 6H), suggesting that ATB$^{0,+}$/*Slc6a14* may be the primary transporter involved in glutamine influx of activated CD8+ T cells in the presence of V-9302. Overexpression of the the rat ATB$^{0,+}$/*Slc6a14* ortholog in preactivated CD8+ T cells resulted in increased ³H-glutamine uptake at similar levels observed with V-9302 treatment (Figure 6I and Supplemental Figure 7, I and J) and conferred greater antigen-specific cytotoxicity to CD8+ OT-I T cells against TNBC tumor cells in vitro (Figure 6J). Together, these results suggest that compensatory upregulation of the glutamine transporter ATB$^{0,+}$/*Slc6a14* in V-9302–treated CD8+ T cells sustains glutamine uptake by T cells despite partial glutamine transporter inhibition, thus maintaining glutamine-dependent T cell cytotoxicity against tumor cells in V-9302–treated tumors. However, compensatory upregulation of glutamine transport was not seen in V-9302–treated TNBC cells, perhaps explaining their exquisite sensitivity to V-9302.

As a requirement to support effector functions, activated CD8+ T cells utilize glutamine to support numerous metabolic processes, including energy production and redox maintenance (21). To assess whether V-9302 impacts T cell energetics, we measured respiration of CD8+ TILs isolated from tumors treated with vehicle or V-9302 (Supplemental Figure 7, K and L). Although glutamine influx is enhanced in the presence of V-9302, the drug does not change the oxygen consumption rate (OCR) of CD8+ TILs. However, ex vivo anti-CD3/anti-CD28–primed CD8+ T cells cultured in V-9302 are more dependent on glutamine to carry out respiration (Supplemental Figure 7M).

Because overall respiration was not affected, we next evaluated if V-9302 and enhanced glutamine influx improved the redox balance of CD8+ T cells by first evaluating glutathione levels. Indeed, anti-CD3/anti-CD28–primed CD8+ T cells cultured in V-9302 had increased glutathione levels compared with the vehicle control (Figure 6K). De novo glutathione synthesis involves ligation of glutamate with the amino acid cysteine by the glutamate-cysteine ligase (GCL). Consistent with increased glutathione production, activated CD8+ T cells cultured in V-9302 showed increases in intracellular cysteine, but not alanine or asparagine, potentially through increased expression of the cysteine transporter, xCT (Figure 6, L and M, and Supplemental Figure 7, N and O). Expression of GCLC, the catalytic subunit of GCL, was also increased in the CD8+ and CD4+ TILs isolated from mice treated with V-9302 (Figure 6N and Supplemental Figure 7P). These increases in glutathione synthetic components following V-9302 treatment were coupled with a decrease in ROS levels of both CD4+ and CD8+ effector T cells (Figure 6O and Supplemental Figure 7Q). Together, these data suggest that V-9302 improves glutamine uptake to support de novo glutathione synthesis and improved redox balance in T cells (Figure 6P).

Discussion

Breast cancer is the most prevalent malignancy in women, encompassing 30% of all new cancer diagnoses, and the second leading cause of cancer-related death. Advances in targeted therapies have improved the survival of ER+ and HER2+ breast cancers, but the prognosis of TNBC, the most aggressive and refractory subtype, remains grim. In search of vulnerabilities for these cancers, TNBCs were found to be dependent on glutamine metabolism (15–19). However, key questions that are critical for drug development remain unanswered, including how tumor cell–derived glutamine metabolism effects immune function and whether inhibitors of GLS and glutamine transporters have an impact on antitumor immunity. In this report, we show that tumor cell–specific glutamine metabolism suppresses tumor-infiltrating T lymphocyte numbers and function, perhaps contributing to the nonresponsiveness of a significant number of TNBCs to immunotherapies observed in the clinic (59). Further, we show that TNBC tumors have increased susceptibility to V-9302, an ASCT2/SNAT2 inhibitor, whereas the CTLs upregulate alternative transporters to sustain glutamine uptake and effector function. Thus, preferential inhibition of glutamine metabolism in tumor cells may represent a promising targeted therapy that enhances antitumor immune responses in TNBC patients.

The tumor microenvironment is a diverse landscape, containing a variety of cell types including tumor cells, immune cell populations (lymphocytes, macrophages, natural killer cells, dendritic cells, myeloid-derived suppressor cells [MDSCs], etc.), fibroblasts, and endothelial cells among others (60, 61). Our data suggest that at least 2 of these populations, tumor cells and T lymphocytes, consume glutamine in the tumor interstitium. However, many of these additional populations also consume and utilize numerous metabolites, including glutamine. For example, M1 macrophage differentiation is favored upon glutamine synthetase inhibition due to greater accumulation of succinate (62), while GLS activity is crucial for endothelial cell proliferation and retinal

angiogenesis in vivo (63). Therefore, the possibility remains that targeting tumor cell glutamine metabolism may alter additional cell populations present in the tumor microenvironment. Given that Rag1 deficiency essentially eliminated the antitumor effects of GLS loss of function, our results suggest that lymphocytes are the primary antitumor effectors in a glutamine-improved tumor microenvironment. However, other immune cells may also be affected by inhibition of glutamine metabolism. For example, the production of α-ketoglutarate via glutaminolysis is important for activation of M2-like macrophages (64), and a prodrug of the glutamine antagonist DON was recently shown to inhibit generation and recruitment of MDSCs (65). Thus, although our findings of improved intratumoral T cell effector functions likely result from the increased glutamine availability to T cells, the potential remains for additional factors that can impact the immune system such as inhibition of suppressive microenvironments by M2-like macrophages and MDSCs.

The role of glutamine metabolism in T cells has been extensively studied in recent years, uncovering its requirement for processes such as proliferation, activation, and differentiation (21–23, 25, 26). Glutamine uptake is massively enhanced upon TCR stimulation in T lymphocytes, and is crucial for their proliferation and cytokine production as glutamine starvation severely limits these downstream processes (22). Recent evidence suggests that preadaptation to glutamine deprivation in vitro enhances $CD8^+$ T cell responses when adoptively transferred in vivo (26, 66, 67), although the underlying mechanisms were not completely understood. Such glutamine deprivation does not reflect conditions within the native tumor microenvironment, where there is a measurable levels of glutamine within the tumor interstitium. Using orthotopic and spontaneous mouse models, we show that loss of GLS in tumor cells increased interstitial glutamine concentrations to near physiological plasma levels (41, 43) and improved overall activation and effector capacity of T lymphocytes, indicating that reduced glutamine in the tumor microenvironment is immunosuppressive. Indeed, activity of effector $CD8^+$ T cells measured by IFN-γ secretion was increased in the presence of elevated glutamine. Likewise, pharmacological inhibition of glutamine transport using V-9302, which suppressed glutamine uptake in tumor cells but had little impact on $CD8^+$ T cells, also improved T cell activation in vivo. Altogether, our results suggest that reducing glutamine metabolism specifically within tumor cells increases glutamine availability within the tumor microenvironment and improves T lymphocyte redox status and activation, supporting the hypothesis of glutamine competition within TNBC tumors.

Amino acid transporters involved in glutamine shuttling have been reported to have varying roles in T cell dynamics. In one report, loss of ASCT2 impaired both Th1 and Th17 differentiation, but enhanced Treg generation without a significant effect on $CD8^+$ T cells (25). Using a different mouse line, a second report showed that ASCT2 deficiency did not affect either B cells or T cells (68), leaving open the question whether pharmacological inhibition of ASCT2 will affect lymphocyte function. While SNAT1/*SLC38A1* and SNAT2/*SLC38A2* expression is increased after TCR-mediated activation (22, 24, 69), little is known regarding their roles in T cell development and function. Despite incomplete understanding of the mechanisms underlying phenotypes

in the ASCT2/*Slc1a5*-deficient mouse models, the facts remain that (a) the glutamine transport inhibitor V-9302 was previously reported to have no impact on CD44 expression on $CD8^+$ T cells in vitro (48), and (b) our in vivo data showed that administration of V-9302 in tumor-bearing mice increased $CD8^+$ T cell activation and increased glutathione synthesis through upregulation of $ATB^{0,+}$. A recent study showed that the glutamine antagonist DON also exhibits differential effects on tumor cells and T cells, but has broad impacts on a variety of metabolic pathways, including glutaminolysis and glycolysis (70), making it difficult to pinpoint the role of glutamine metabolism in these cell populations in vivo. Thus, the drug V-9302 or its analogs may represent an alternative approach to preferentially target tumor cells while sparing antitumor T cells.

Pharmacologically targeted therapies are capable of impacting multiple cell populations found within the tumor microenvironment as well as the human body. Due to the glutamine-addicted nature of TNBC cells, a great deal of attention has recently been focused on targeting glutamine metabolism as a means to therapeutically target these aggressive tumors. For example, the GLS inhibitor CB-839 is undergoing clinical trial testing for numerous solid tumors. Although CB-839 as a single-agent therapy has not produced promising results, the pleiotropic roles of glutamine suggest that CB-839 may be a good candidate for combination therapy to induce synthetic lethal effects on cancer cells. However, it may be that throttling the supply of glutamine would be needed in some settings. Our findings that V-9302 inhibits glutamine transport into tumor cells while enhancing Th1 and $CD8^+$ T cell activation represents a potential therapeutic strategy to preferentially target glutamine metabolism in tumor cells. Our work suggests that strategies preferentially targeting glutamine metabolism in glutamine-addicted tumor cells may represent a more advantageous therapeutic approach to treating TNBC.

Methods

Flow cytometry. Immune cell populations were identified via flow cytometry from respective dissociated whole tumor cell suspensions or cultured cells. To assess mTORC1 signaling and ROS, C57BL/6 mouse (Jackson Laboratories) splenocytes were activated with anti-mouse CD3e (Tonbo, 70-0031; 1.25 μg/mL) and anti–mouse CD28 (Tonbo, 70-0281; 1.25 μg/mL) while treated with vehicle (DMSO) or V-9302 (10 μM) for 48 hours. For phos-flow cytometry, cells were collected in the presence of phosphatase inhibitor and stained with 7-AAD (10 μg/mL). Fixed cells were permeabilized in cold methanol, and phospho-S6RP (Ser235/236; Cell Signaling Technology, 2211) was indirectly detected (Alexa Fluor 647–goat anti-rabbit secondary, 1:200, Invitrogen, A21244). T cells were identified by extracellular staining of mouse CD45, CD3e/TCRβ, CD4, and CD8a (Supplemental Figure 3A and Supplemental Table 3). To detect ROS, cells were incubated with 5 μM H_2-DCFDA (Invitrogen, D399) at 37°C for 30 minutes. Dead cells were excluded using Ghost Dye Violet 510 (Tonbo, 13-0870).

For tumor immune analyses, tumors were harvested, weighed, and dissociated in RPMI-1640, 5% FBS, collagenase IA (1 mg/mL; Sigma-Aldrich, C9891), and DNase I (0.25 mg/mL; Sigma-Aldrich, DN25) for 30 minutes at 37°C. To obtain single-cell suspensions, digested tissue was filtered through a 70 μm cell strainer and red blood cells were lysed using ACK lysis buffer. For detection of IFN-γ, 1×10^6 to

2×10^6 cells were stimulated in RPMI-1640 containing 5% FBS, phorbol 12-myristate 13-acetate (PMA) (50 ng/mL; Sigma-Aldrich, P8139), ionomycin (1 μg/mL; Sigma-Aldrich, I0634), and GolgiPlug protein transport inhibitor (1:1000; BD, 555029) for 4 hours at 37°C. Dead cells were excluded from analysis by staining with Ghost Dye Violet 450 (Tonbo, 13-0863) or Violet 510, as indicated. Following blocking with anti-CD16/32 (mouse Fc block), extracellular staining was achieved against the following proteins: CD45, CD45.2, CD3e, TCRβ, CD4, CD8a, CD25, CD69, CD44, CD62L, CD127, and/or CD107a (Supplemental Table 3). Intracellular staining for GZMB, IFN-γ, IL-4, IL-17A, and GCLC was accomplished using a Cytofix/Cytoperm solution kit (BD, 554714) on paraformaldehyde-fixed cells, per the manufacturer's directions. GCLC was indirectly detected by Alexa Fluor 647–goat anti-rabbit secondary antibody as described above. To detect FoxP3, fixed cells were stained using the FoxP3/Transcription Factor Staining Kit (Tonbo, TNB-0607), as directed. Isolated splenocytes or tumor cell suspensions were used for unstained, compensation, fluorescence minus one (FMO), and isotype controls, where appropriate. Flow cytometry data were obtained on a BD Fortessa using BD FACSDiva software and analyzed using FlowJo software.

To assess T cell infiltration, E0771(OVA) sgLacZ (WT) or sgGls3 (GLS^{KO}) (5×10^5 cells) were bilaterally implanted into the number 4 mammary fat pads of 6- to 8-week-old C57BL/6 female mice (Jackson Laboratories or Taconic as indicated in the figure legends). OT-I splenocytes were isolated from 4-week-old female OT-I mice (Jackson Laboratories) and activated with SIINFEKL peptide (1 μg/mL; Invivogen) for 72 hours. Following activation, CD8$^+$ OT-I T cells were isolated using mouse CD8a microbeads (Miltenyi Biotec, 130-045-201) as described by the manufacturer and then transduced with MiG retrovirus to overexpress GFP. GFP$^+$CD8$^+$ OT-I T cells (3×10^6) were adoptively transferred via retro-orbital injection into tumor-bearing mice 8 days following tumor implantation. After 3 days, tumors were harvested, processed, and analyzed by flow cytometry as described above. Dead cells were excluded from analysis by staining with Ghost Dye Violet 510. CD8$^+$ OT-I cells were detected by GFP.

Tumor interstitial fluid metabolic assays. Tumor interstitial fluid was collected from tumors as previously described (71), with some changes. Briefly, tumors were harvested, weighed, and then washed with 1× PBS to remove excess blood from the tumor surface. Excess PBS was removed by carefully blotting with a Kimwipe. Tumors were placed in a 20 μm pluriStrainer (pluriSelect) mounted in a microcentrifuge tube, then centrifuged at 100g at 4°C for 30 minutes. The cleared interstitium was deproteinated and glutamine was detected using the Glutamine Detection Assay Kit (Abcam, ab197011) according to the manufacturer's instructions. Known glutamine standards were used to calculate concentrations.

Intracellular amino acid assays. To assess intracellular cysteine, alanine, and asparagine levels, anti-CD3/anti-CD28–activated CD8$^+$ T cells freshly isolated from C57BL/6 mice were cultured in vehicle or V-9302, as described above. Equal numbers of cells were homogenized and analyzed for cysteine (Abcam, ab211099), alanine (Sigma-Aldrich, MAK001), or asparagine (BioVision, K736) as described by the manufacturers. Tumor cell intracellular glutamate concentrations were determined using the Glutamate Assay Kit (Sigma-Aldrich, MAK004-1KT) as previously described (6) with some changes. Briefly, control or GLS loss-of-function E0771 single-cell clones were cultured in glutamine-free, serum-free DMEM for 24 hours. Cells were then stimulated with L-glutamine (2.5 mM) for 15 minutes. Fluorescence or absorbance was measured using the BioTek Synergy HT plate reader. Amino acid concentrations were determined using known standards and are reported as fold change using the appropriate control.

IFN-γ cytokine secretion by Tc1 cells in response to glutamine. CD8$^+$ T cells were purified from spleens of C57BL/6 mice and activated with plate-bound anti-CD3 (2.5 μg/mL) and soluble anti-CD28 (2.5 μg/mL) in the presence of irradiated antigen-presenting cells (APCs). CD8$^+$ T cells were cultured at 1×10^6 cells per mL in 2 mL on 12-well plates with 2×10^6 irradiated APCs and fed rh-IL2 (20 ng/mL) every other day. Cells were split on day 2 into 6-well plates and cultured for a total of 4 days. On day 4, cultures were harvested, counted, and equal numbers of cells were replated at a density of 2×10^6 cells/mL in media with indicated concentrations of glutamine, reactivated with fresh anti-CD3 and anti-CD28 (1.25 μg/mL each), and cultured overnight in a 37°C CO$_2$ incubator. Culture supernatants were collected after 18–24 hours of culture for anti–IFN-γ cytokine sandwich ELISA, as previously described (72), using anti–mouse IFN-γ (BD, 551216) and anti–mouse IFN-γ–biotin (BD, 554410) as capture and detection antibodies, respectively.

Cytotoxicity assay. For in vitro cytotoxicity assays, E0771(OVA) or E0771 (1×10^4 per well) were plated in triplicate as indicated and allowed to attach for 6 hours. Tumor cells were cocultured with SIINFEKL-activated WT or MiG-ATB$^{0,+}$–transduced CD8$^+$ OT-I cells (2×10^3 per well, resulting in 5:1 tumor cell to CD8$^+$ cell ratio) in T cell media in the absence or presence of V-9302 (10 μg/mL), as indicated, for 48 hours. Cytotoxicity was measured using the LDH Cell-mediated Cytotoxicity Assay (Thermo Fisher Scientific), according to the manufacturer's directions. Percentage cytotoxicity was calculated following correction for spontaneous LDH release from tumor cells and CD8$^+$ T cells alone and based on maximal LDH release for each condition.

Glutamine uptake assay. C57BL/6 or OT-I mouse CD8$^+$ cells were isolated, activated, and modified, as indicated. Human PMBCs were activated with functional grade anti–human CD3 (1 μg/mL; Invitrogen, 16-0038) and anti–human CD28 (1 μg/mL; Invitrogen, 16-0289), with supplementation of human IL-2 (20 U/mL) for 48 hours and extracted using human CD8 microbeads (Miltenyi Biotec, 130-045-201) as directed. E0771 (3.5×10^4 per well) or HCC1806 (1.0×10^4 per well) cells were plated in at least duplicate in a 96-well plate, and cells were allowed to attach overnight, as indicated. Glutamine uptake was measured in tumor or CD8$^+$ cells in isolation or tumor/CD8$^+$ cell cocultures (1:1) as described previously (48), with a few alterations including omission of BCH except where specified. Briefly, tumor cells and/or CD8$^+$ cells were washed 3 times in uptake buffer (10 mM HEPES pH 7.4, 137 mM NaCl, 5.1 mM KCl, 0.77 mM KH$_2$PO$_4$, 0.71 mM MgSO$_4$, 1.1 CaCl$_2$, and 10 mM D-glucose). CD8$^+$ T cells were resuspended in assay buffer containing V-9302 (10 μM), MeAIB (10 mM) (Sigma-Aldrich, M2383), BCH (500 μM) (Cayman Chemical, 15249), and/or αMT (100 μM) (Sigma-Aldrich, M8377), as indicated, along with the appropriate vehicles. For coculture assays, CD8$^+$ cells were directly added to tumor cells. Following a 15-minute incubation at 37°C, 1 μCi of L-[2,3,4-3H]-glutamine (American Radiolabeled Chemicals, ART-0149) was added, and cells were incubated an additional 15 minutes. Suspended CD8$^+$ cells were removed, and both tumor and T cells were washed in uptake buffer separately. Cells were lysed with 1N NaOH, and radioactivity was measured using a Beckman LS 6500 Scintillation counter for 1 min-

ute and recorded as counts per minute (CPM). Treated samples were normalized to the appropriate controls.

Glutathione assay. CD8+ T cells were activated and cultured in the presence of vehicle or V-9302, as described above. Glutathione was measured using the GSH-Glo Glutathione Assay (Promega, V6911) according to the manufacturer's instructions. Luminescence was measured using the BioTek Synergy HT plate reader. Glutathione concentrations were determined from a known standard.

Quantitative real-time polymerase chain reaction. E0771 or 4T1 cells (5×10^5) were plated and treated with vehicle or 10 μM V-9302 for 48 hours. Likewise, isolated activated primary mouse splenocytes were treated with vehicle or V-9302, followed by isolation of CD8+ cells as described above. RNA was collected using the RNeasy kit (Qiagen), and cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturers' directions. Samples were amplified in triplicate using SYBR Green PCR Master Mix (Thermo Fisher Scientific), 10 pmol of primer (Supplemental Table 4), and 20 ng of cDNA per reaction with the StepOnePlus (Applied Biosystems). Quantitation was performed using the $\Delta\Delta C_t$ method.

Gene expression and survival data set analysis. TCGA Breast Adenocarcinoma (BRCA) database (HiSeqV2), downloaded January 19, 2018 from the UCSC Xena platform (73) (https://xena.ucsc.edu), was searched for mRNA expression and z-scores were calculated based on all tumor samples. The average z-scores for glutaminase (*GLS*), asparagine synthetase (*ASNS*), glutamine-fructose-6-phosphate transaminase 1 (*GFPT1*), ASCT2 (*SLC1A5*), SNAT1 (*SLC38A1*), SNAT2 (*SLC38A2*), and ATB[0,+] (*SLC6A14*) comprise the glutamine metabolism gene signature (GMGS). The average z-score of the glycolysis genes *ALDOA*, *ENO1*, *GAPDH*, *GPI*, *HK1*, *LDHA*, *PFKM*, *PFKL*, *PGAM1*, *PKM2*, and *TPI1* as well as the GLUT1 transporter (*SLC2A1*) generated the glycolysis gene signature. To avoid confounding data from highly glycolytic tumors, the BLBC samples (PAM50, ref. 74) with expression profiles signifying the greatest dependence on glutamine metabolism ("glutamine metabolism high") were identified based on glutamine metabolism and glycolysis gene signatures (top 12.5%). All other BLBC tumors were classified as having "mixed metabolism." A CTL gene signature was calculated by averaging the z-scores of *GZMA*, *GZMB*, *PRF1*, and *IFNG* expression.

For survival analyses, the glutamine metabolism gene signature (*GLS*, 211414_at; *ASNS*, 205047_s_at; *GFPT1*, 202722_s_at; *SLC1A5*, 208916_at; *SLC38A1*, 218237_s_at; *SLC38A2*, 220924_s_at; *SLC6A14*, 219795_at) and the CTL gene signature (*GZMA*, 205488_at; *GZMB*, 210164_at; *PRF1*, 214617_at; *IFNG* 210354_at) gene expression and overall survival (OS) and distant metastasis-free survival (DMFS) data from basal breast cancer tumors were downloaded from KM plot (75) (http://www.kmplot.com). Automatic cutoff was used to define "high" versus "low" expression, with cutoff values defined as followed: *GLS* (OS: 57, range = 2–587) (DMFS: 43, range = 2–242), *ASNS* (OS: 1560, range = 109–13,370) (DMFS: 902, range = 33–13,370), *GFPT1* (OS: 1726, range = 354–5352) (DMFS: 1138, range = 377–5352), *SLC1A5* (OS: 380, range = 131–2039) (DMFS: 709, range = 131–2039), *SLC38A1* (OS: 3704, range = 94–16,075) (DMFS: 2817, range = 370–14,915), *SLC38A2* (OS: 2469, range = 288–13,482) (DMFS: 2976, range = 751–7711), *SLC6A14* (OS: 899, range = 1–8829) (DMFS: 920, range = 3–10,359), *GZMA* (OS: 239, range = 17–4627) (DMFS: 272, range = 46–4627), *GZMB* (OS: 289, range = 7–6030) (DMFS: 295, range = 18–6030), *PRF1* (OS: 201, range = 46–6453) (DMFS: 203, range = 56–6453), and

IFNG (OS: 58, range = 1–668) (DMFS: 68, range = 2–665). Characterization of "high" expression of the CTL gene signature required "high expression" classification of at least 2 genes, while characterization of "high" expression of the glutamine metabolism gene signature required "high expression" classification of at least 4 genes. Samples were then categorized based on the glutamine metabolism gene signature or *GLS* expression and the CTL gene signature.

Statistics. For survival curve analysis, the Mantel-Cox method was used for log-rank analysis of all groups, with the Bonferroni correction utilized for multiple comparisons where applicable. Hazard ratios (HRs) and 95% confidence intervals were calculated for single comparisons using the log-rank test.

For dose-response analyses, nonlinear fit was applied to both CB-839– and V-9302–treated cells. To directly assess differences between CB-839 and V-9302, unpaired Student's t test was applied at the indicated points.

All plots and statistical analyses were completed using Graph-Pad Prism software. Data from independent assays (dot) as well as summary data (mean with SEM) are shown. For tumor experiments, data are reported from 1–2 independent experiments, with each dot representing a single animal. For comparisons between 2 groups, unpaired or paired Student's t tests were performed as indicated. For multiple comparisons, 1- or 2-way analysis of variance (ANOVA) was performed with post hoc analysis using Tukey's, Dunnett's, Holm-Sidak's, or Sidak's multiple-comparison tests, as indicated. Outliers were identified using the ROUT method ($Q = 5\%$). All statistical tests were completed as 2-tailed, and P less than 0.05 was considered statistically significant, except where corrected for multiple comparisons as indicated. All error bars are SEM.

Study approval. All mouse procedures were performed under protocols approved by Vanderbilt University's Institutional Animal Care and Utilization Committee (IACUC).

Author contributions

DNE and JC conceptualized the project. DNE, VMN, ALR, SW, YH, LCK, and YP performed the experiments and analyzed the data. SHC, QW, SZ, and MRB provided technical and conceptual support. JCR, HCM, and RSC provided critical reagents. DNE, ALR, RSC, MRB, and JC wrote the manuscript.

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