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Cystathionine-gamma-lyase overexpression in T cells enhances antitumor effect independently of cysteine autonomy

Melanie Lancien^{1,2} | Lucile Gueno^{1,2} | Sonia Salle^{1,2} | Emmanuel Merieau^{1,2} |
 Gaelle Beriou^{1,2} | Tuan H. Nguyen^{1,2} | Ahmed Abidi^{1,3} | Nahzli Dilek⁴ |
 Pierre Solomon¹ | Jeremie Poschmann¹ | Olivier Michielin^{4,5,6} | Romain Vuillefroy de Silly⁵ |
 Bernard Vanhove^{1,7} | Cedric Louvet¹ 

¹Inserm, Centre de Recherche en Transplantation et Immunologie, UMR 1064, ITUN, Université de Nantes, Nantes, France

²LabEx IGO "Immunotherapy, Graft, Oncology", Nantes, France

³Faculty of Sciences, Tunis, University of Tunis El Manar, Tunis, Tunisia

⁴Molecular Modeling Group, SIB Swiss Institute for Bioinformatics, Lausanne, Switzerland

⁵The Ludwig Institute for Cancer Research, University of Lausanne, Epalinges, Switzerland

⁶Department of Oncology, University of Lausanne and Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland

⁷Xenothera, Nantes, France

Correspondence

Cedric Louvet, CRTI Inserm 1064, Nantes, France.

Email: cedric_louvet@dfci.harvard.edu

Bernard Vanhove, Xenothera, Nantes, France.

Email: bernard.vanhove@xenothera.com

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Abstract

T cells could be engineered to overcome the aberrant metabolic milieu of solid tumors and tip the balance in favor of a long-lasting clinical response. Here, we explored the therapeutic potential of stably overexpressing cystathionine-gamma-lyase (CTH, CSE, or cystathionase), a pivotal enzyme of the transsulfuration pathway, in antitumor CD8⁺ T cells with the initial aim to boost intrinsic cysteine metabolism. Using a mouse model of adoptive cell transfer (ACT), we found that CTH-expressing T cells showed a superior control of tumor growth compared to control T cells. However, contrary to our hypothesis, this effect was not associated with increased T cell expansion *in vivo* or proliferation rescue in the absence of cysteine/cystine *in vitro*. Rather than impacting methionine or cysteine, ACT with CTH overexpression unexpectedly reduced glycine, serine, and proline concentration within the tumor interstitial fluid. Interestingly, *in vitro* tumor cell growth was mostly impacted by the combination of serine/proline or serine/glycine deprivation. These results suggest that metabolic gene engineering of T cells could be further investigated to locally modulate amino acid availability within the tumor environment while avoiding systemic toxicity.

KEYWORDS

adoptive cell transfer, amino acid, cysteine, metabolism, T cell

1 | INTRODUCTION

Impressive results have been obtained in patients with cancer by engineering T cells with cloned T-cell receptors (TCRs) or chimeric

antigen receptors. However, while most of the attention has been focused on tumor antigen specificity/escape, signal tuning, and safety of the infused T cells,¹ additional therapeutic weapons are needed to overcome evasion, especially in solid cancers where

Melanie Lancien and Lucile Gueno contributed equally to this work

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the tumor microenvironment can exert strong immunosuppressive effects. Arming T cells with original ectopic genes could promote specific pathways to counteract the hostile metabolic environment that mostly results from the energetic demands of malignant cells.² For example, previous studies focused on enabling diverse intrinsic advantages to T cells from glucose uptake and metabolism³ to oxidative stress,⁴ mitochondrial biogenesis,⁵ cholesterol metabolism⁶ or intracellular K⁺ regulation.⁷ There is also growing evidence that amino acid availability critically regulates T cell expansion and function as recently documented for arginine (Arg),⁸ serine (Ser),⁹ and others.¹⁰

In our previous work on novel mechanisms of allograft tolerance,¹¹ we found that cystathionine- γ -lyase (CTH or also named CSE, CGL, or cystathionase), a pivotal enzyme in the transsulfuration pathway,¹² was downmodulated during immune tolerance in monocytes and dendritic cells (DCs) and controlled interleukin-12 (IL-12) expression. These findings drew our attention to the immunoregulatory role of cysteine (Cys) metabolism that is likely shared between transplant and tumor immunology. T cells use the transsulfuration pathway poorly to metabolize their own Cys and are highly dependent on the extracellular availability of cystine (Cys₂, oxidized dimeric form of Cys), imported through the Cys₂/glutamate (Glu) antiporter xCT, only expressed after cell activation.¹³ Alternatively, T cells import Cys provided by antigen-presenting cells such as macrophages and DCs through their ASC neutral amino acid transporter^{14,15} or through thioredoxin-mediated reduction of Cys₂ to Cys.¹⁶ Levring et al reported that T cell proliferation, rather than activation, is strictly dependent on Cys/Cys₂ uptake¹⁷ that is mostly needed for subsequent production of the major antioxidant molecule glutathione (GSH).^{15,18} It has been proposed that this metabolic dependency is exploited by immunosuppressive cells including regulatory T cells (Tregs)^{19,20} or tumor-induced myeloid-derived suppressive cells (MDSCs) that sequester Cys.²¹

In this study, we tested whether CTH overexpression in antitumor CD8⁺ T cells could rewire the transsulfuration pathway and possibly confer Cys production autonomy. Although we found that this approach allowed a better control of tumor growth in a mouse model of adoptive cell transfer (ACT), it did not prevent T cell vulnerability to Cys/Cys₂ deprivation but, instead, was associated with reduced concentration of three other amino acids within the tumor.

2 | MATERIALS AND METHODS

2.1 | Mice

C57BL/6 (Ly5.2⁺) mice were purchased from Janvier. OT-I Ly5.1 homozygous mice were obtained by intercrossing OVA-specific TCR-transgenic OT-I mice (C57BL/6-Tg(TcraTcrb)1100Mjb/Crl; Charles River) with Ly5.1 mice (B6.SJL-PtprcaPepcb/BoyCrl; Charles River). All mice used for experiments were between 8 and 12 weeks of age and kept under specific pathogen-free conditions. Experimental procedures were carried out in strict accordance with the protocols

approved by the Committee on the Ethics of Animal Experiments of Pays de la Loire and authorized by the French Government's Ministry of Higher Education and Research.

2.2 | Quantification of metabolites

Tumor interstitial fluid (TIF) was isolated from tumors as previously described.²² Amino acids, lanthionine, pyruvate, and hydrogen sulfide were quantified in plasma, T cell culture medium, and/or TIF by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as detailed in Appendix S1 and in Table S1.

2.3 | Vectors and ecotropic retrovirus production

The coding sequence (excluding the stop codon) of the mouse *Cth* cDNA sequence (NM_145953.2) was codon-optimized, synthesized, and cloned into the MSGV gamma-retroviral plasmid preceding the sequences encoding the T2A "self-cleavage" peptide and the Thy1.1 (CD90.1) marker. Control retrovirus encoded Thy1.1 alone. These plasmids (2.2 μ g) were separately mixed with pCL-Eco plasmid (1.3 μ g) and 23 μ L Lipofectamine 2000 (Thermo Fisher Scientific) in OptiMEM (Gibco) for 20 minutes at room temperature (RT). This mix was then added to each well of 6-well plates to transfect HEK 293T cells in DMEM base (Gibco) supplemented with 10% heat-inactivated FBS (Eurobio), nonessential amino acids, 1 mmol/L sodium pyruvate, (Thermo Fisher Scientific), 10 mmol/L HEPES (Gibco), and 2 mmol/L glutamine (Gln) (Sigma; without penicillin/streptomycin for the transfection and later steps). The medium was replaced after 6 hours; 2 days later, supernatants were collected and filtered (0.45 μ m) to be freshly used for T cell infection.

2.4 | Mouse T cell infection

Six-well plates were coated with 5 μ g/mL anti-CD3 (clone 145.2C11; BD Biosciences) in PBS for 2 hours at 37°C followed by three washes with PBS. CD8⁺ T cells were enriched using the CD8a⁺ T Cell Isolation Kit II (Miltenyi Biotec) from the pooled spleen and lymph nodes of OT-I Ly5.1 mice and incubated (4×10^6 cells in 4 mL/well) for 48 hours in anti-CD3-coated plates in DMEM base (Gibco) supplemented with 10% heat-inactivated FBS (Eurobio), nonessential amino acids, 1 mmol/L sodium pyruvate, penicillin/streptomycin (Thermo Fisher Scientific), 10 mmol/L HEPES (Gibco), 2 mmol/L Gln (Sigma), 50 μ mol/L β -mercaptoethanol (Sigma), 0.5 μ g/mL anti-CD28 (clone 37.51, BD Biosciences) and 50 IU/mL recombinant human IL-2 (Proleukine). The day before infection, wells of a 6-well plate were coated with 50 μ g/mL RetroNectin (Takara Bio) for 16 hours at 4°C. On the day of infection, RetroNectin was replaced by 2% BSA (Sigma) in PBS for 30 minutes at 37°C followed by a wash using 25 mmol/L HEPES. Activated CD8⁺ T cells were collected and adjusted to 2×10^6 cells/mL. One milliliter was then

added per well of RetroNectin-coated plates along with 3 mL of retrovirus-containing supernatant. Protamine sulfate (Sigma) and IL-2 were added at final concentrations of 4 $\mu\text{g}/\text{mL}$ and 50 IU/mL, respectively. Spinoculation was carried out (2000 g, lowest acceleration and no brake) for 1 hour at 30°C. The cells were incubated for 4 hours at 37°C. Two milliliters of supernatant was then replaced with fresh IL-2-containing medium. After 24 hours, the cells were used for adoptive transfer in vivo or expanded in vitro for analysis.

2.5 | Quantitative PCR

Total RNA was isolated using RNeasy Mini Kit or Micro Kit (Qiagen). Reverse transcription was carried out using M-MLV Reverse Transcriptase and random primers following manufacturer's instructions (Thermo Fisher Scientific). Quantitative PCR was carried out using ViiA 7 Real-Time PCR System and Fast SYBR Green Master Mix reagent (Applied Biosystems, Thermo Fisher Scientific). Gene-specific primers were designed over different exons to prevent amplification of genomic DNA. Gene expression was normalized to *Gapdh* and expressed in arbitrary units using the $2^{-\Delta\Delta\text{Ct}}$ method. For *Cth* quantification, degenerate primers were designed to target both the endogenous and the retroviral codon-optimized *Cth* cDNA sequence. The following oligonucleotides were used: mouse *Gapdh* forward, GGTGAAGGTCGGTGTGAACGG; mouse *Gapdh* reverse, TCGCTCCTGGAAGATGGTGAT; amplicon size, 232; mouse *Cth* forward, YTGAGCGGCTTCTGCC; mouse *Cth* reverse, GCCSGGAAGTCTCTGCTT; and amplicon size, 138 (Y = C or T; S = C or G).

2.6 | Immunoblot analysis

Two days after T cell infection, protein was extracted using RIPA buffer (Cell Signaling Technology) supplemented with protein inhibitor cocktail (Sigma). Protein concentration for each sample was determined using BCA assay (Interchim). Equal amounts of protein were loaded on 4%-15% polyacrylamide gel (Bio-Rad), separated by SDS-PAGE and transferred using semidry western blot to a nitrocellulose membrane (GE Healthcare Life Science). The membrane was blocked for 2 hours at RT in TBST 3% BSA, probed with anti-CTH Ab (clone S51, dilution 1/1000, Abnova) at 4°C overnight, then washed and incubated with goat anti-mouse HRP secondary Ab (dilution 1/10 000, Jackson ImmunoResearch Laboratories) at RT 1 hour. The membrane was then washed and incubated for 5 minutes at RT with the Western Pierce ECL Chemiluminescent Substrate (Thermo Fisher Scientific) blot images were acquired with a Luminescent Image analyzer LAS-4000 (FujiFilm). The membrane was then stripped using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) and used for subsequent detection of β -actin using a mouse anti- β -actin Ab (clone 8H10D10, dilution 1/1000, Cell Signaling Technology).

2.7 | In vivo model of antitumor ACT

B16-OVA tumor cells were recovered from log phase in vitro growth and 1×10^6 cells were injected s.c. in 50 μL cold PBS into the flank skin of recipient female C57BL/6 mice. B16-OVA cells tested negative for mycoplasma and could be specifically killed by OVA-specific CD8⁺ T cells in vitro. Tumor size was measured in a blind fashion with a caliper and expressed as the area based on two perpendicular diameters. At day 10 (for long-term tumor growth assessment) or 14 (for subsequent immunoprofiling or metabolic analysis), mice bearing tumors of a similar size were randomly divided into three groups (six to eight mice per group for each experiment) and received total body irradiation (TBI; 600 cGy). Infected OT-I CD8⁺ T cells (0.5×10^6) were then injected i.v. Interleukin-2 (6×10^4 IU) was injected i.p. on the day of ACT and over the two following days. Mice were monitored daily and were killed when tumor size reached 300 mm², or if a rapid weight loss was observed (15% loss in body weight from last maximum weight measurement), or if there were signs that an animal was in morbid condition.

2.8 | Flow cytometry analysis

Dead cells were marked for exclusion using Fixable Viability Dye eFluor 506 (eBioscience) or DAPI (Thermo Fisher Scientific). Fc receptors were blocked before all staining using CD16/32 Ab (BD Biosciences). Cells were subsequently stained with fluorochrome-conjugated mAbs listed in Table S2. Intracellular staining was carried out using the eBioscience Foxp3/Transcription Factor Staining Buffer Set. For cytokine analysis, cells were stimulated with PMA (50 ng/mL) and ionomycin (1 $\mu\text{g}/\text{mL}$; Sigma) in the presence of GolgiStop (BD Biosciences) for 5 hours. Samples were acquired on a FACSCanto II cytometer (BD Biosciences) and data were analyzed using FlowJo software (TreeStar). Specific mean fluorescence intensity of the indicated marker was determined by subtracting the background fluorescence obtained from a separate control staining of the population of interest. Absolute cell number was determined using 123count eBeads (Thermo Fisher Scientific).

2.9 | In vitro T cell proliferation assay

To modulate Cys/Cys₂ availability in the culture medium, DMEM, high glucose, no Gln, no methionine (Met), no Cys₂ (Gibco) was supplemented as described above. Then 200 $\mu\text{mol}/\text{L}$ Met hydrochloride solution (Sigma) and the indicated concentrations (0-200 $\mu\text{mol}/\text{L}$) of Cys₂ hydrochloride solution (Sigma) were added. Purified CD8⁺ T cells were first stimulated in 200 $\mu\text{mol}/\text{L}$ Cys₂ complete medium (day 0) and retrovirally infected (day 2) as described above. After 2 days of expansion (day 4), the cells were labeled with Cell Proliferation Dye (CPD) eFluor 670 (eBioscience), resuspended in 200, 20, 5, or 0 $\mu\text{mol}/\text{L}$ Cys₂ complete medium

and distributed in a 96-well plate (0.5×10^6 cells per well). Live cell number and CPD fluorescence dilution were assessed by flow cytometry analysis 2 days later.

2.10 | In vitro cytotoxicity assay and restimulated T cell phenotype

B16-OVA cells were plated in a 96-well flat-bottom plate (1.5×10^4 cells/well in 200 μ L complete RPMI medium). After 24 hours, OT-1 CD8⁺ T cells (transduced 2 days before) were added at different ratio with fresh IL-2-containing medium. The plate was spun at 400 g for 1 minute and incubated at 37°C for 18 hours. B16-OVA cells were trypsinized and the number of live CD8⁺ DAPI⁻ annexin V⁻ cells was assessed by flow cytometry using 123count eBeads (Thermo Fisher Scientific). T cell phenotype was analyzed from a different plate 6 hours after incubation with B16-OVA tumor cells (antigen-specific restimulation) at 1:2.5 (tumor : T cell) ratio.

2.11 | In vitro effect of selective amino acid deprivation on B16-OVA tumor cells

To modulate Ser, proline (Pro), and glycine (Gly) availability in the culture medium, we used a custom-made RPMI medium without Gln, Ser, Pro, or Gly (Cell Culture Technologies) supplemented with 10 mmol/L HEPES (Gibco), 2 mmol/L Gln (Sigma), 1 mmol/L sodium pyruvate, penicillin/streptomycin (Thermo Fisher Scientific), and 0.5% heat-inactivated FBS (Eurobio). L-serine (Ser), L-proline (Pro), and Gly hydrochloride solutions (Sigma) were added as indicated. B16-OVA cells were first expanded in complete medium containing Ser, Pro, and Gly at a saturating concentration (200 μ M). The cells were then seeded (2×10^3 cells at 200 μ L/well) in a flat-bottom 96-well plate at the indicated concentrations of Ser, Pro, and Gly. Four days later, the number of live DAPI⁻ annexin V⁻ cells was assessed by flow cytometry using 123count eBeads (Thermo Fisher Scientific).

2.12 | Statistical analysis

Statistical analyses were carried out using GraphPad Prism using the Mann-Whitney test and, for survival rates, using the log-rank (Mantel-Cox) test. *P* values less than .05 were considered significant.

3 | RESULTS

3.1 | Rationale and overexpression of CTH in T cells

Based on a model positing a critical requirement of Cys/Cys₂ deprivation and extracellular redox modulation concurrently induced

by cancer cell metabolic needs,²³ MDSCs²¹ or Tregs¹⁹ (Figure 1A), we first sought to determine the level of Cys/Cys₂ and the other proteogenic amino acids in the B16-OVA mouse melanoma model. We compared plasma and TIF using LC-MS/MS (Figure 1B). As expected, Cys/Cys₂ concentration was among the lowest amino acids found in plasma and TIF, along with glutamic acid (Glu). Interestingly, similarly to a recent study using a mouse model of pancreatic ductal adenocarcinoma,²² only Glu and Gly appeared enriched in TIF. Conversely, the levels of Gln, lysine (Lys), aspartate (Asp), Arg, tryptophan (Trp), Met, and Cys/Cys₂ were significantly decreased in TIF compared to plasma. Thus, these results are consistent with a decreased availability of Cys/Cys₂ within the tumor microenvironment.

CTH is catalyzing the last enzymatic step in the transsulfuration pathway to obtain Cys from Met (Figure 1C). We reasoned that CTH overexpression in T cells could boost the functionality of this pathway and enhance their anti-tumor effect in vivo by bypassing Cys deprivation. To test this hypothesis, we designed a bicistronic gamma-retroviral vector encoding CTH and the reporter surface protein Thy1.1 (Figure 1D). Thy1.1 expression alone was used in the control retrovirus. In vitro infection of activated mouse CD8⁺ T cells routinely reached ~40%-60% as measured by Thy1.1 expression, without reproducible differences between CTH and control infections over multiple experiments (Figure 1E). We confirmed that CTH induction was achieved both at the mRNA (Figure 1F) and protein (Figure 1G) level, as compared with virtually no endogenous expression of CTH in control T cells. Importantly, this induction did not significantly impact the expression of various T-cell markers (Figure 1H) including inhibitory checkpoint receptors or the potential of these cells to produce γ -interferon (IFN γ)⁺ and tumor necrosis factor- α (TNF α)⁺ cells (Figure 1I). Thus, stable overexpression of CTH can be achieved in T cells without hampering their proliferation and phenotype at least in the short term in vitro.

3.2 | Cystathionine-gamma-lyase expression enhances the effect of antitumor ACT independently of T cell accumulation in the tumor

To test the effect of CTH-expressing T cells in vivo, we used a model of ACT in which B16-OVA melanoma-bearing mice are treated with OVA-specific CD8⁺ T cells from OT-I TCR-transgenic mice (Figure 2A). In our experimental conditions, we found that a conditioning regimen (TBI and IL-2 injections) at the time of ACT was required to observe a transient effect of ACT on tumor growth (Figure 2B). Adoptive cell transfer with CTH-expressing T cells allowed a modest but significant delay in tumor growth, compared to control ACT (Figure 2C,D) that was also associated with a prolongation of mouse survival (Figure 2E).

Next, we examined the fate of the infused OT-I CD8⁺ T cells in vivo following ACT using the CD45.1 congenic marker (Figures 3A and S1). Surprisingly, CTH expression did not induce increased

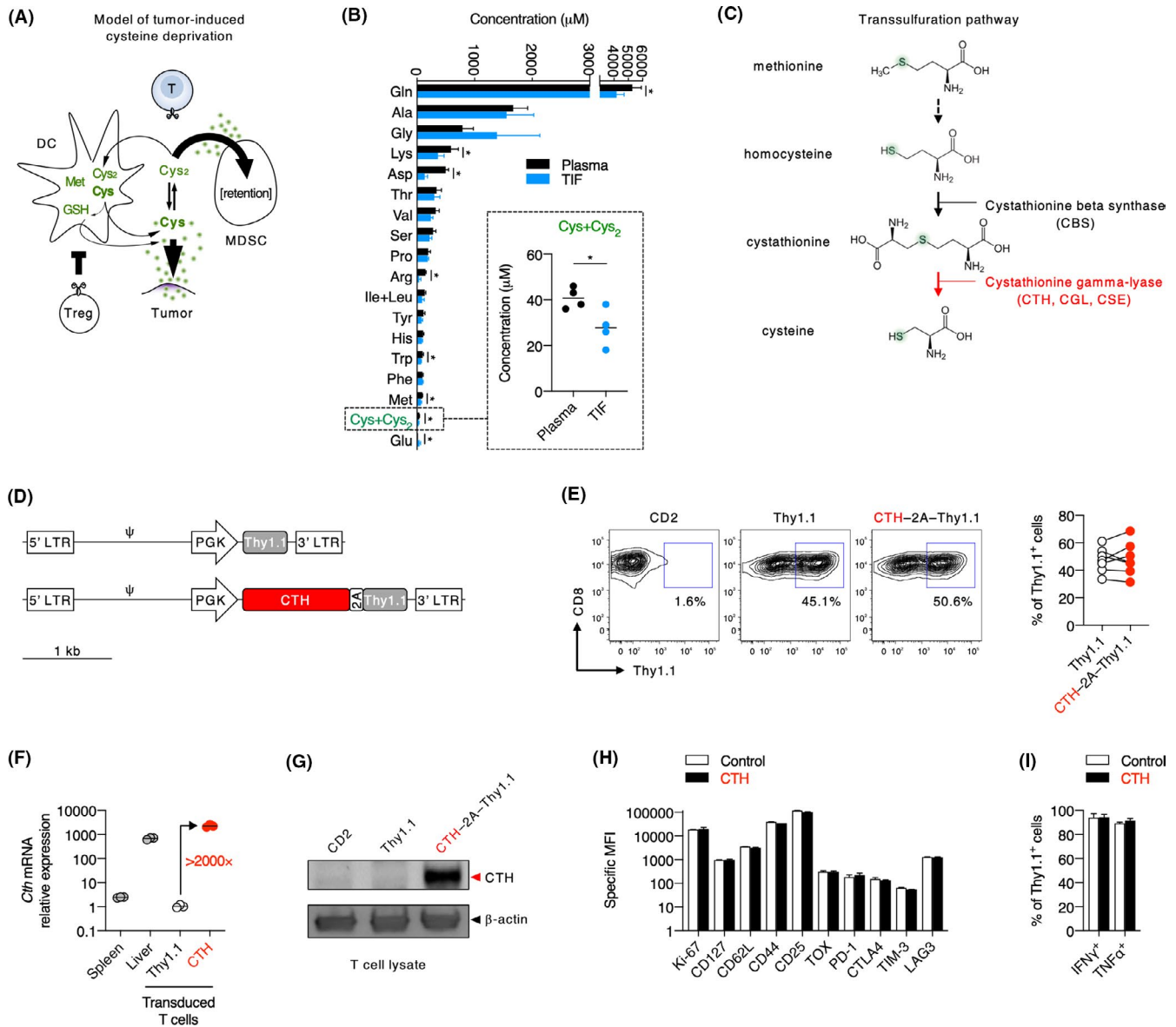


FIGURE 1 Cystathionine gamma-lyase (CTH) overexpression in T cells. A, Model positing the alteration of cysteine (Cys) metabolism and deprivation in the tumor environment as a major mechanism of T cell dysfunction. Dendritic cells (DC) generate Cys by importing and reducing cystine (Cys₂) or by converting methionine (Met) to Cys through the transsulfuration pathway. Regulatory T cells (Treg) suppress Cys accumulation in the extracellular compartment and interfere with glutathione (GSH)-mediated redox remodeling by DCs. Myeloid-derived suppressor cells (MDSC) sequester Cys₂ without returning Cys in the extracellular compartment. Tumor cells consume Cys for their metabolic needs. B, Comparison of amino acid concentrations measured by liquid chromatography-tandem mass spectrometry between the plasma and the tumor interstitial fluid (TIF) of B16-OVA melanoma-bearing mice. **P* < .05. C, Simplified view of the transsulfuration pathway. Dotted arrow represents multistep conversions. D, Retroviral constructs for the expression of the surface reporter protein Thy1.1 alone (control) or along with CTH under the PGK promoter. E, Representative flow cytometry analysis of Thy1.1 expression showing the efficacy of mouse CD8⁺ T cell transduction 2 days after infection (left panel) and quantification of seven independent experiments (right panel). F, RT-qPCR analysis of *Cth* mRNA expression in mouse CD8⁺ T cells following *Cth*-expressing retroviral infection as compared to endogenous expression in control T cells, spleen, and liver of naïve mice. G, Western blot analysis showing overexpression of CTH protein in *Cth*-transduced T cells as compared to control T cells overexpressing CD2 or Thy1.1 alone. H, Flow cytometry analysis of the indicated markers. Ki-67 and TOX expression was assessed following cell permeabilization. I, Flow cytometry analysis of γ -interferon (IFN γ)⁺ and tumor necrosis factor- α (TNF α)⁺ cells following restimulation and cell permeabilization. Data (means \pm SD) are representative of two independent experiments

numbers of total CD45.1⁺ or transduced Thy1.1⁺ T cells in the tumor or draining lymph nodes (dLN; Figure 3B). Accordingly, the proliferation marker Ki-67 was similarly expressed in CTH-expressing

and control T cells (Figure 3C). Furthermore, CTH expression did not modulate the level of expression of T cell markers including CD44, the regulator of exhaustion program TOX, or the inhibitory

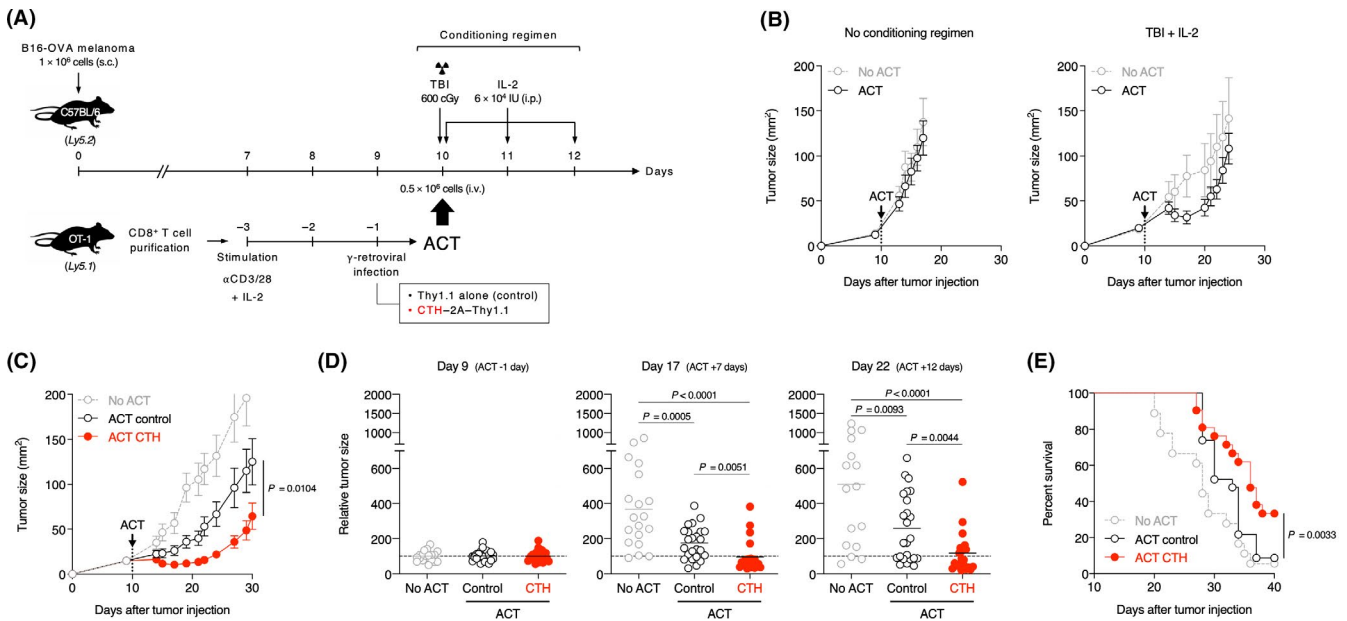


FIGURE 2 In vivo antitumor effect of infused cystathionine gamma-lyase (CTH)-expressing CD8⁺ T cells. A, Schematic representation and timeline of the preclinical model of antitumor T cell therapy. B, Conditioning regimen requirement for revealing a transient adoptive cell transfer (ACT) effect on tumor growth (mean area ± SEM). C, Rates of tumor growth represented over time in untreated mice (No ACT: total body irradiation [TBI] + interleukin-2 [IL-2] alone) or following ACT of OT-I CD8⁺ T cells transduced with Thy1.1 alone (control) or along with CTH ($n = 6-8$ mice per group). Data show one experiment representative of three independent experiments. Statistical difference between control and CTH ACT is shown using area under curve (AUC) comparison. D, Relative tumor growth data normalized from three independent experiments (No ACT, $n = 19$; ACT control, $n = 24$; ACT CTH, $n = 23$) where the mean of tumor size of the No ACT group at day 9 was set at 100. E, Mouse survival in the indicated groups and pooled from three independent experiments

receptor PD-1 (Figure 3C). Finally, the production of IFN γ , TNF α , or granzyme B following in vitro restimulation was neither significantly enhanced nor diminished by CTH expression (Figure 3D).

We also examined whether CTH-expressing cells could influence endogenous immune cells. However, we did not find any significant alteration, notably in the Treg (FOXP3⁺) or the myeloid-derived suppressor cell (CD11b⁺Ly6C/G⁺) populations (Figures 3E-H and S1).

Thus, CTH expression in T cells results in a superior antitumor effect following ACT that cannot be attributed to increased proliferation of the infused T cells or to an alteration of endogenous immunoregulatory cell numbers.

3.3 | Expression of CTH does not improve the cytotoxic function of T cells in vitro

Although CTH overexpression does not appear to boost the proliferative capacity of T cells, it could enhance their cytotoxic function. We set up a cytotoxic assay by incubating transduced OT-I CD8⁺ T cells with B16-OVA cells (Figure 4A). First, we observed that CTH expression did not change the expression of selective T cell markers following short-term restimulation (Figure 4B). Second, control and CTH-expressing T cells showed similar dose-dependent cytotoxic activity against B16-OVA cells (Figure 4C). Thus, it is likely that the in vivo effect of T-cell CTH overexpression on tumor growth control

does not result from a directly improved cytotoxic function of transduced T cells.

3.4 | Expression of CTH does not prevent the inhibitory effect of Cys/Cys₂ deprivation on T cell proliferation

The absence of effect of CTH expression on T cell proliferation in vivo prompted us to test whether CTH overexpression in T cells could overcome Cys/Cys₂ deprivation in vitro. Indeed, although the transsulfuration pathway actually exists in T cells,¹³ Levring et al showed that Met cannot substitute for Cys or Cys₂ to enable proliferation.¹⁷ In our experimental settings (Figure 5A), differing from Levring et al using naïve T cells, Cys₂ was removed from the medium 4 days after stimulation to allow T cell infection (day + 2) and expression of the transgenes, well after proliferation had begun. Hence, under these conditions, the need for Cys₂ in the medium could be less critical. However, Cys₂ deprivation still strongly reduced the number of live cells analyzed 2 days later, in a dose-dependent manner (Figure 5B). Importantly, CTH expression did not prevent this inhibition of proliferation (Figure 5B-D) or change the concentration of Met or Cys/Cys₂ measured in the supernatant at the end of the culture (Figure 5E). Taken together, these results indicate that CTH overexpression is not sufficient to confer Cys production autonomy

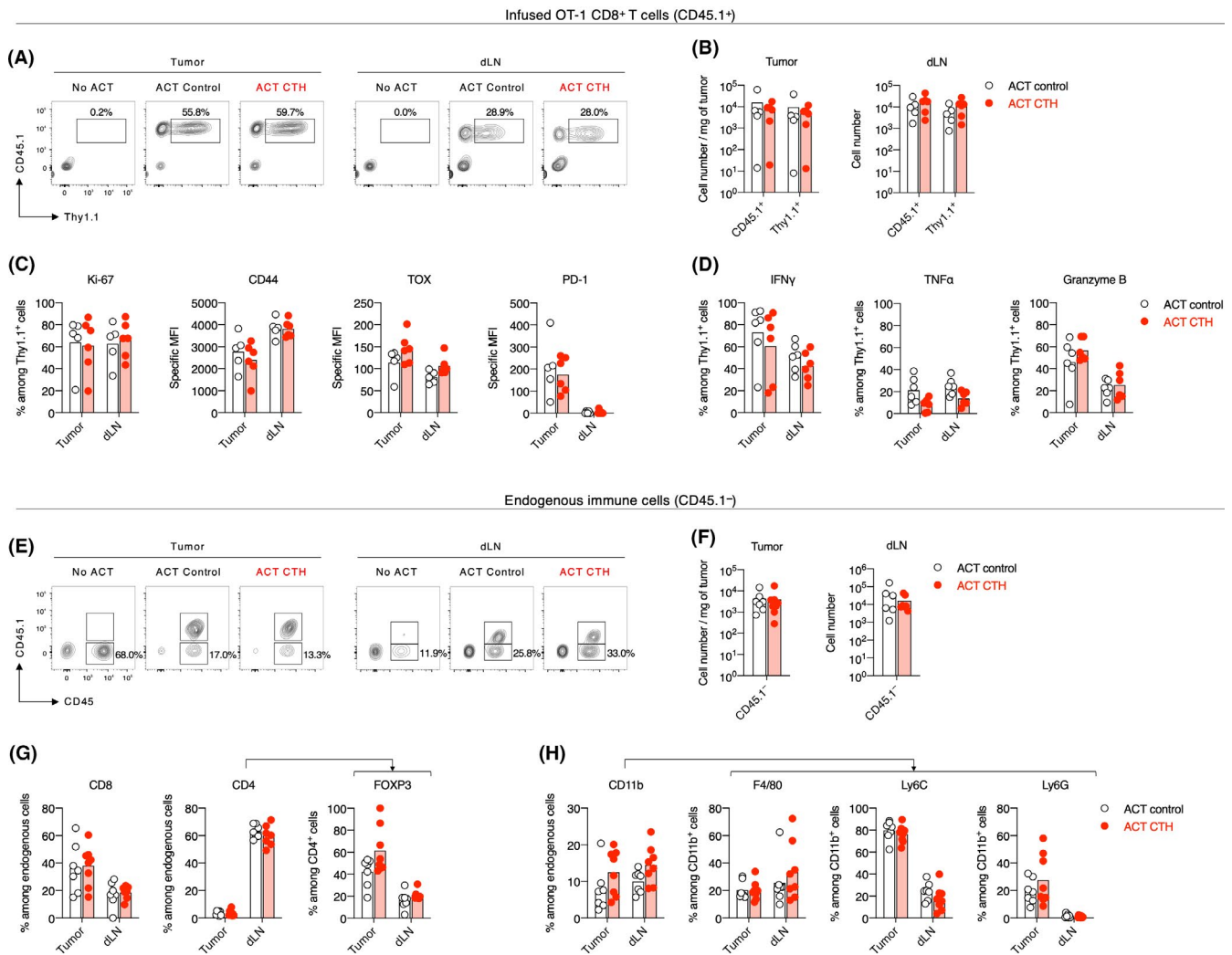


FIGURE 3 In vivo analysis of infused T cells and endogenous immune cells. Adoptive cell transfer (ACT) was carried out 14 (instead of 10) days after tumor cell inoculation to allow recovery and analysis by flow cytometry of significant numbers of cells in each tumor. Tumor and draining lymph nodes (dLN) were harvested 1 week later for analysis. A-D, In vivo tracking and analysis of adoptively transferred CD45.1⁺ (OT-1) T cells by flow cytometry. A, Thy1.1 expression shows control and cystathionine gamma-lyase (CTH)-expressing cells within the infused CD45.1⁺ (OT-1) T cells. B, Quantification of total CD45.1⁺ or Thy1.1⁺ cells in tumors (cell numbers per milligram of tumor) and in the dLNs. Dots show values from individual mice ($n = 5-6$ mice per group) and bars show means. C, Expression of the indicated markers among CD45.1⁺ Thy1.1⁺ cells. D, Expression of γ -interferon (IFN γ), tumor necrosis factor- α (TNF α), and granzyme B among CD45.1⁺ Thy1.1⁺ cells following in vitro restimulation with PMA-ionomycin. Data are representative of two independent experiments. E-H, In vivo analysis of endogenous T and myeloid cells. E, Representative flow cytometry analysis of endogenous hematopoietic CD45.1⁻ cells. F, Quantification of endogenous CD45⁺CD45.1⁻ cells in tumors (cell numbers per milligram of tumor) and in the dLNs. G, H, Percentages of endogenous T and myeloid cells as indicated by their respective markers. Data are representative of two independent experiments

to T cells and avoid subsequent proliferation blockade induced by Cys/Cys₂ deprivation.

3.5 | Expression of CTH in T cells modulates levels of Gly, Ser, and Pro within TIF following ACT

To determine whether CTH expression could impact the concentration of proteogenic amino acids other than Cys, we analyzed their concentrations following 7 days of in vitro culture, both in T cell lysates and in supernatants (Figure 6A). We did not observe any difference between control and CTH-expressing T cell lysates

(Figure 6B, left panel), and only barely detectable although significant changes in supernatants for some amino acids including Glu, Pro, isoleucine + leucine (Ile + Leu), and phenylalanine (Phe; Figure 6B, right panel).

Next, we sought to directly analyze the amino acid concentrations in vivo, either in the plasma or in the TIF, by comparing mice treated with control and CTH-expressing antitumor T cells (Figure 6C). Adoptive cell transfer (along with conditioning regimen) was carried out at day +14 after tumor cell inoculation to allow sizeable tumors for TIF recovery. In the plasma, CTH expression did not alter the concentration of the 20 amino acids, or only very modestly for threonine (Thr) and Lys (Figure 6D, left panel). In contrast, TIF analysis revealed that

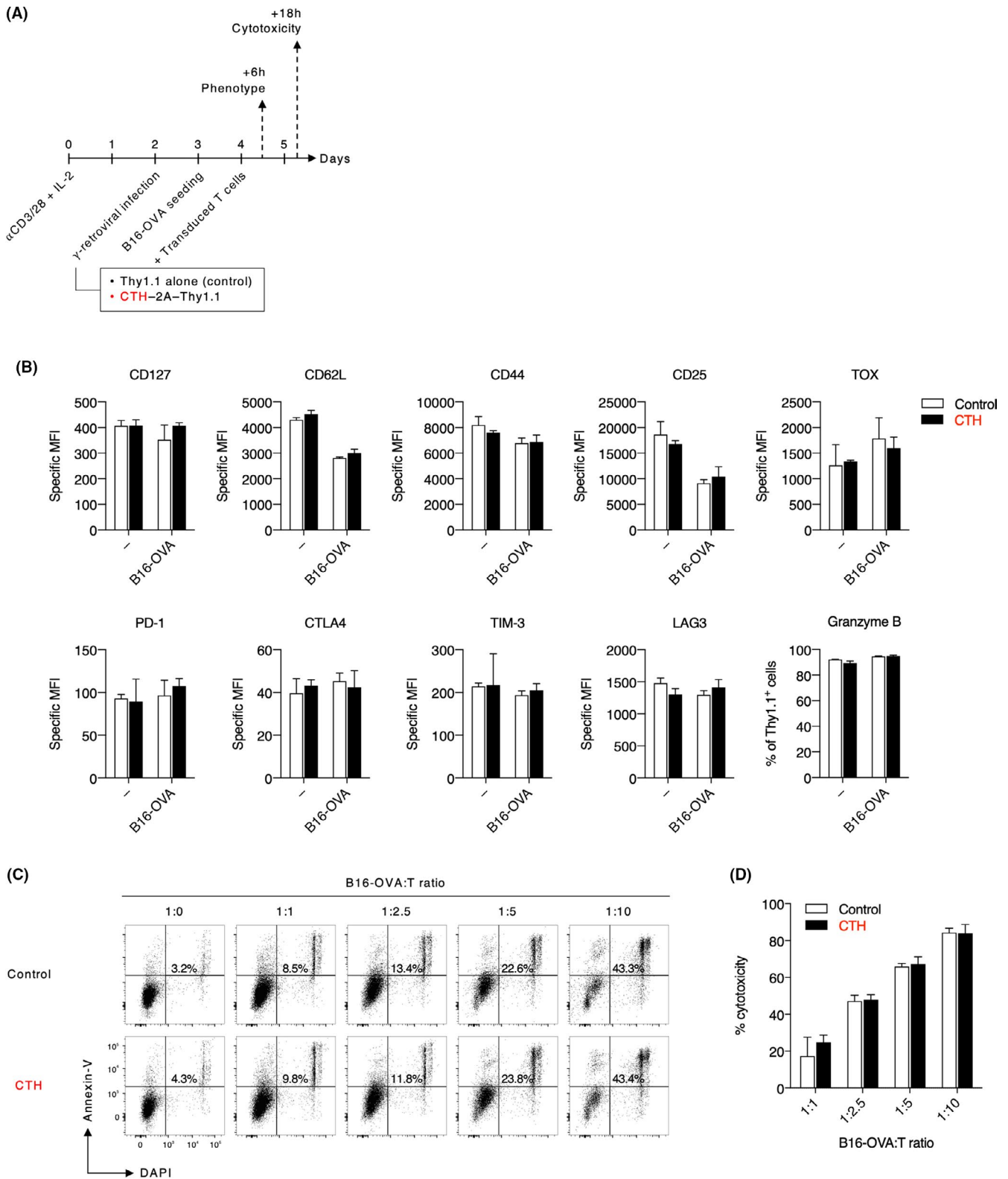


FIGURE 4 Cystathionine gamma-lyase (CTH) overexpression does not affect in vitro T-cell cytotoxicity. A, Schematic representation of the cytotoxicity assay using transduced CD8⁺ OT-1 T cells and B16-OVA tumor cells as target cells. B, After 6 h of incubation of T cells with or without B16-OVA cells (1:2.5 ratio, B16-OVA:T), T cell phenotype was analyzed by flow cytometry using the indicated markers. TOX and granzyme B expression was assessed following cell permeabilization. Data (means \pm SD) are representative of two independent experiments. C, D, After 18 h of incubation of T cells with B16-OVA cells at the indicated ratios, live CD8⁺ DAPI⁻ annexin V⁻ cells were quantified by flow cytometry and the percentage of cytotoxicity was deduced. IL-2, interleukin-2; MFI, mean fluorescence intensity

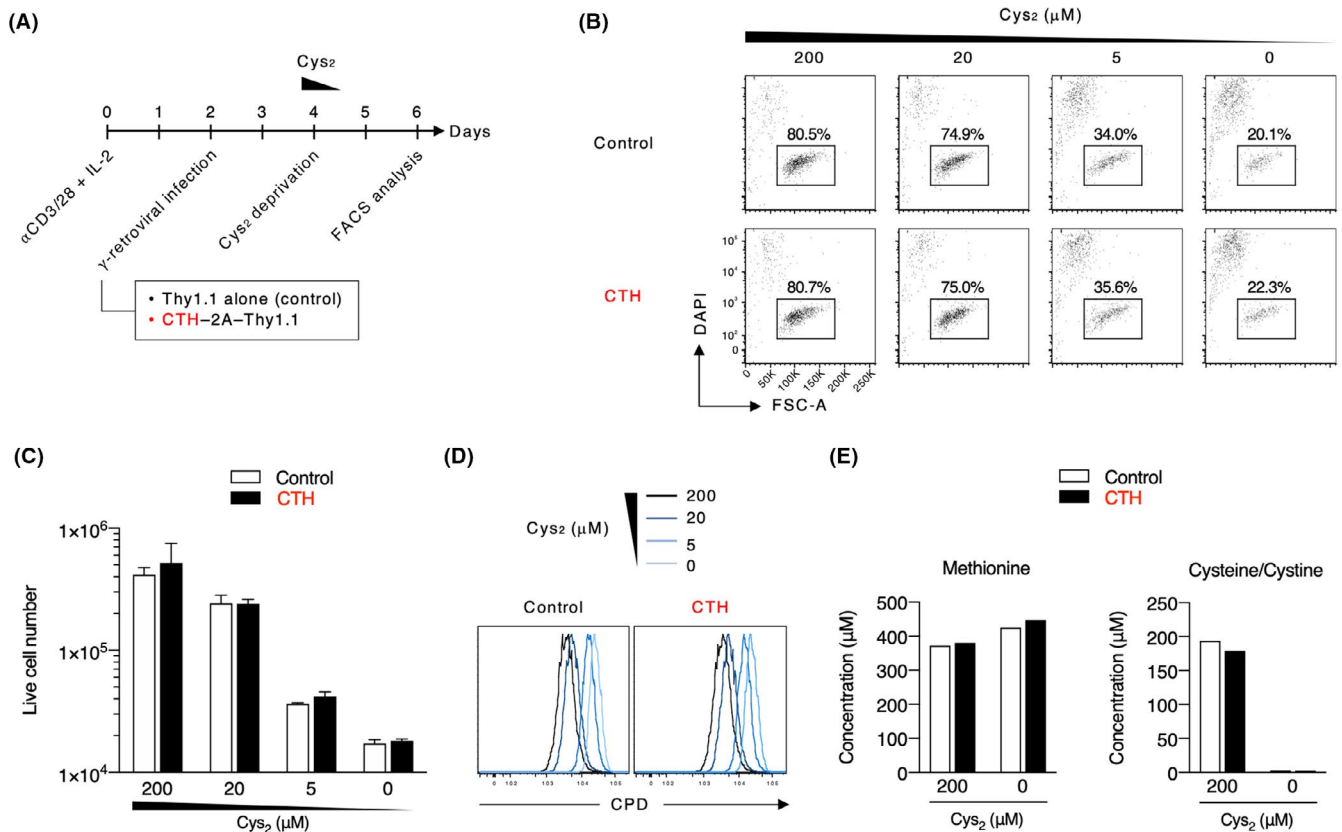


FIGURE 5 Cystathionine gamma-lyase (CTH) overexpression in T cells does not rescue proliferation inhibition induced by cystine (Cys₂) deprivation in vitro. A, Schematic representation of T cell infection (day 2 after stimulation) by control or CTH-encoding retrovirus followed by Cys₂ removal from the medium (day 4). Cell viability and proliferation were assessed after 2 days (day 6). B, Flow cytometry analysis showing identification of DAPI⁺ live cells. C, D, Absolute live cell quantification (mean \pm SD) (C) and cell proliferation dye (CPD) fluorescence (D), inversely reflecting cell proliferation, in the presence of the indicated concentrations of Cys₂ in the medium. E, Quantification by liquid chromatography-tandem mass spectrometry of methionine and cysteine (Cys)/Cys₂ at the end of the culture (day 6) in the supernatants of wells supplemented or not with Cys₂ at day 4. Data are representative of at least two independent experiments. FSC-A, forward scatter area

three amino acids, namely Gly, Pro, and Ser, were specifically less abundant in mice treated with CTH-expressing T cells compared to mice treated with control T cells (Figure 4D, right panel). Although Cys or Met were not significantly impacted, the concentration of Gly, Pro, and Ser decreased by 82%, 78%, and 72%, respectively (Figure 6E).

3.6 | Serine/Pro or Ser/Gly combinations are required for in vitro tumor cell survival

The decrease of Gly, Ser, and Pro in the tumors of CTH ACT-treated mice suggested a direct causal effect of reduced amino acid availability on tumor growth. Interestingly, the concomitant reduction of Gly, Ser, and Pro concentrations significantly reduced tumor cell survival in vitro (Figure 7A,B), thus mirroring the decreased tumor growth observed in CTH ACT-treated mice. Furthermore, Ser appeared as the dominant amino acid as only its absence combined with the absence of Pro or Gly showed the most important impact on tumor cell growth (Figure 7C,D).

Together, these data suggest that CTH-expressing cells could modulate the availability of selective amino acids, notably Ser,

within the tumor environment to ultimately affect tumor cell survival. Interestingly, RNA sequencing analysis did not reveal any significantly differentially expressed genes between CTH-expressing compared to control T cells, including amino acid transport, Ser, or Gly metabolism genes (Figure S2), suggesting that a possible T cell metabolic rewiring induced by CTH overexpression would not occur at a transcriptional level.

4 | DISCUSSION

Although the addition of N-acetyl-L-cysteine on antitumor T cells in vitro has previously been shown to enhance their survival and functional capacities,²⁴⁻²⁶ the present study is the first to aim at intrinsically targeting the Cys metabolism in T cells by genetic manipulation. Strikingly, although the overexpression of CTH, a key enzyme of the transsulfuration pathway, in T cells enhanced the effect of ACT in a robust preclinical solid tumor model, our data strongly suggest that it was not associated with increased autonomous production of Cys. In this regard, it is likely that appropriate levels of other enzymes such as the cystathionine-beta-synthase (CBS) or cofactors

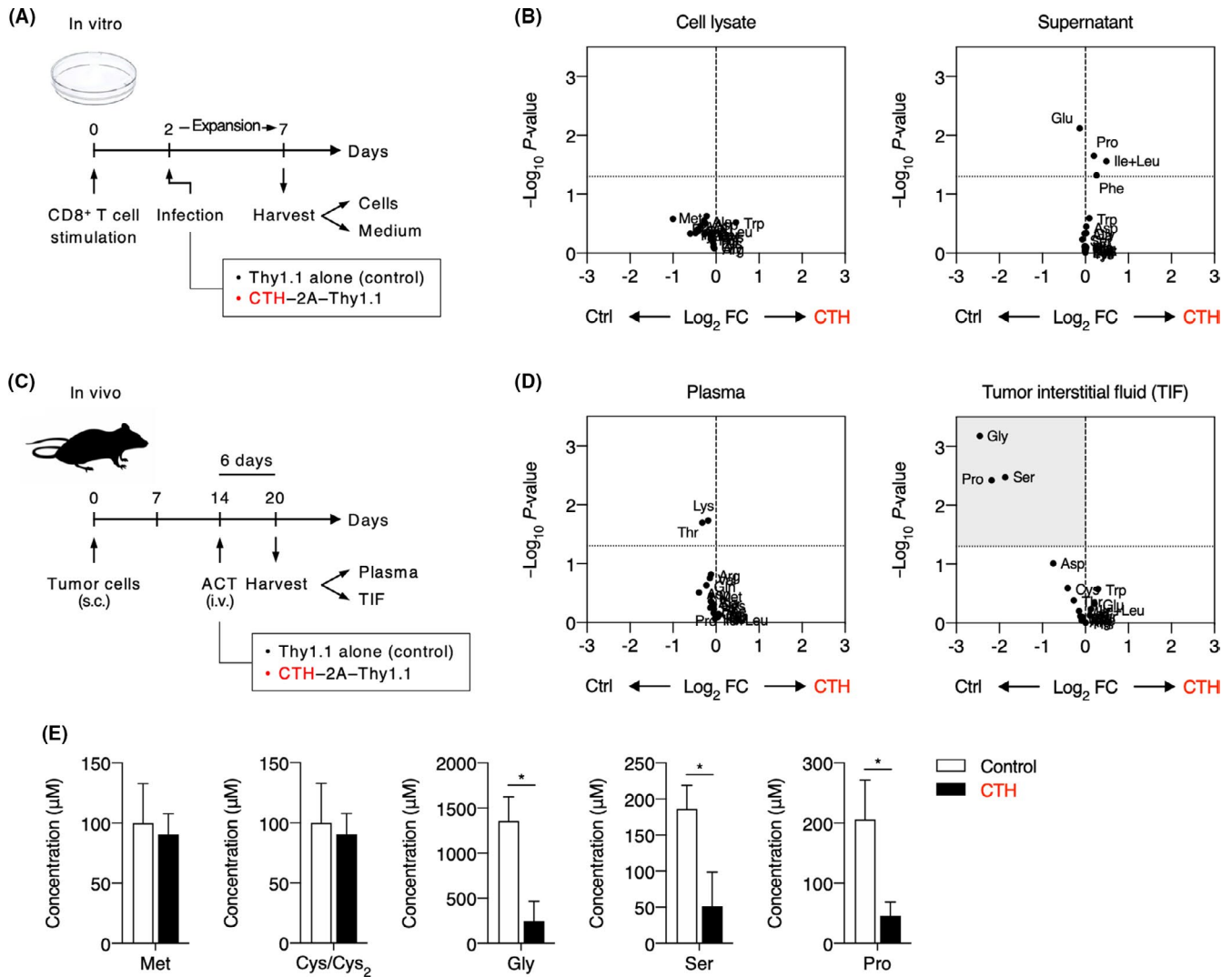


FIGURE 6 Analysis of amino acid changes induced by cystathionine gamma-lyase (CTH) expression in vitro and in vivo. A, Schematic representation of T cell stimulation and infection by control (Ctrl) or CTH-encoding retrovirus followed by in vitro expansion in complete medium supplemented with 500 IU/mL interleukin-2 (IL-2). Cells and supernatants were collected on day 7. B, Volcano plot depicting the \log_2 fold change (FC) in proteogenic amino acid concentration in cell lysates (left panel) or supernatants (right panel) between Ctrl and CTH-expressing T cell in vitro cultures. C, Schematic representation of B16-OVA melanoma cell inoculation followed by indicated adoptive cell transfer (ACT; along with total body irradiation + IL-2 injections). Six days after ACT, circulating plasma and tumor were harvested ($n = 4$ mice per group). D, Volcano plot depicting the \log_2 FC in proteogenic amino acid concentration in the plasma (left panel) or tumor interstitial fluid (TIF; right panel) between Ctrl and CTH ACT-treated mice. E, Concentration (mean \pm SD, four mice in each group) of indicated amino acids in TIF of control and CTH ACT-treated mice. * $P < .05$

are required to develop a fully functional transsulfuration pathway. Further investigation will be needed to determine whether such a metabolic pathway reconstitution is actually achievable in T cells.

Surprisingly, we found that three amino acids other than Cys, namely Gly, Ser, and Pro, showed a decreased concentration specifically in the TIF of mice treated with CTH-expressing T cells. The fact that this was not observed in vitro could be explained by the saturating concentration of the amino acids in the medium. Alternatively, it is possible that a more complex mechanism develops in T cells in a longer term under the tumor physiologic niche in vivo and that the unique interplay of tumor, stromal, myeloid, and T cells within the tumor environment is necessary. Interestingly, because Ser is used

with homocysteine by CBS to produce cystathionine, a major substrate of CTH, the diminution of this amino acid in the TIF could be a consequence of an altered transsulfuration pathway in T cells. Moreover, Ser is a critical immunometabolite for effector T cell expansion and can supply Gly for nucleotide biosynthesis.⁹ Dietary restriction of Ser and Gly has been shown to significantly impact tumor growth in various mouse models of cancer²⁷ and rapidly proliferating cancer cells could be rendered highly vulnerable to decreased availability of Gly.²⁸ Finally, alteration of Pro concentration in the TIF could also contribute to tumor growth inhibition as the pharmacological modulation of its catabolism has been shown to impair cancer cell metastasis in vivo.²⁹

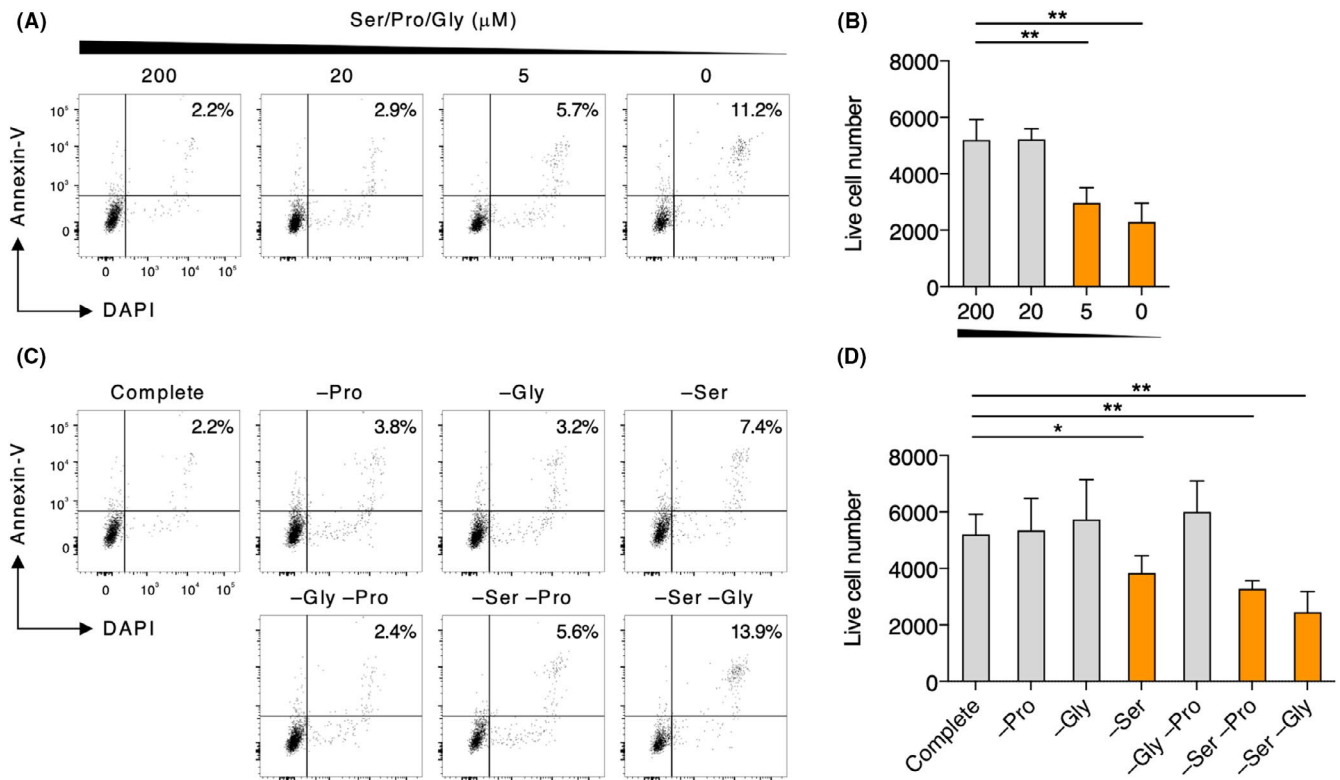


FIGURE 7 In vitro effect of serine (Ser), proline (Pro), and glycine (Gly) deprivation on tumor cell viability. A, B, B16-OVA cells were cultured for 4 days using the indicated concentrations for each of the three amino acids Ser, Pro, and Gly. Live DAPI⁺annexin V⁺ cells were quantified by flow cytometry. Percentages show increased cell death induced by the concomitant deprivation of Ser, Pro, and Gly. C, D, B16-OVA cells were cultured for 4 days with different deprivation combinations: absence of only one (-Pro, -Gly, or -Ser) or two amino acids at the time. Live cells were quantified as described above. Data (means \pm SD, four replicates per condition) are representative of three independent experiments. * $P < .05$; ** $P < .01$

Interestingly, our in vitro data reinforce the hypothesis of a critical role of Ser, along with Pro or Gly, for B16-OVA tumor cell survival. In-depth in vitro experiments using CTH-expressing T cells under nonsaturating concentrations of Ser, Pro, and Gly could possibly demonstrate the capacity of these cells to induce proximate selective amino acid deprivation. Importantly, given that the present study does not provide intracellular mechanistic insights, investigating a potential intracellular metabolic rewiring in T cells—most likely posttranscriptionally—will be key to better understand how CTH overexpression could ultimately lead to this deprivation within the tumor microenvironment. Similarly, a causal relationship between the decrease of these three amino acids and reduced tumor size in CTH ACT-treated mice remains to be established and could be achieved using supplemented and/or altered diets.

In conclusion, although the precise intrinsic metabolic changes that are induced remain to be elucidated, our data suggest that CTH overexpression in T cells results in an enhanced control of tumor growth, conceivably through a significant alteration of the metabolic milieu rather than increased T cell proliferation. These results are in line with promising strategies aimed at starving cancer cells of critical amino acids. In fact, tumor growth suppression by depletion of serum Cys/Cys₂ was elegantly achieved by

Cramer et al using a pharmacologically optimized CTH, termed cyst(e)inase,³⁰ an effect more recently confirmed in other mouse cancer models and explained by the induction of ferroptosis, an iron-dependent nonapoptotic cell death.^{31,32} The use of T cells endowed to deliver such biologics could prove to be a very potent therapeutic approach and reduce the risk of toxicity caused by systemic and chronic drug treatment.

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DISCLOSURE

The authors have no conflict of interest.

ORCID

Cedric Louvet  <https://orcid.org/0000-0002-5668-2448>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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