

Downregulation of phosphoserine phosphatase potentiates tumor immune environments to enhance immune checkpoint blockade therapy

Zhi-Peng Peng,^{1,2} Xing-Chen Liu,¹ Yong-Hao Ruan,¹ Da Jiang,¹ Ai-Qi Huang,¹ Wan-Ru Ning,¹ Ze-Zhou Jiang,¹ Limin Zheng ,^{1,2} Yan Wu ¹

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Z-PP and X-CL contributed equally.

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¹Guangdong Province Key Laboratory of Pharmaceutical Functional Genes, MOE Key Laboratory of Gene Function and Regulation, School of Life Sciences, Sun Yat-Sen University, Guangzhou, China

²State Key Laboratory of Oncology in Southern China, Collaborative Innovation Center for Cancer Medicine, Sun Yat-sen University Cancer Center, Guangzhou, China

Correspondence to

Dr Yan Wu;
wuyan32@mail.sysu.edu.cn

Professor Limin Zheng;
zhenglm@mail.sysu.edu.cn

ABSTRACT

Background Effects of immune checkpoint blockade (ICB) treatment in hepatocellular carcinoma (HCC) are limited. The current study explored the possibility of exploiting tumor metabolic switches to enhance HCC sensitivity to immune therapies.

Methods Levels of one-carbon (1C) metabolism and the expression of phosphoserine phosphatase (PSPH), an upstream enzyme of 1C pathway, were evaluated in paired non-tumor and tumor tissues from HCC. Underlying mechanisms mediating the role of PSPH in regulating the infiltration of monocytes/macrophages and CD8⁺ T lymphocytes were studied through both in vitro and in vivo experiments.

Results PSPH was significantly upregulated in tumor tissues of HCC and its levels were positively correlated with disease progression. PSPH knockdown inhibited tumor growth in immunocompetent mice, but not in those with macrophage or T lymphocyte deficiencies, indicating the pro-tumor effects of PSPH were dependent on both immune components. Mechanistically, PSPH facilitated monocytes/macrophages infiltration by inducing the production of C-C motif chemokine 2 (CCL2), while at the same time reduced CD8⁺ T lymphocytes recruitment through inhibiting the production of C-X-C Motif Chemokine 10 (CXCL10) in tumor necrosis factor alpha (TNF- α)-conditioned cancer cells. Glutathione and S-adenosyl-methionine were partially involved in regulating the production of CCL2 and CXCL10, respectively. shPSPH (short hairpin RNA) transfection of cancer cells enhanced tumor sensitivity to anti-programmed cell death protein 1 (PD-1) therapy in vivo, and interestingly, metformin could inhibit PSPH expression in cancer cells and mimic the effects of shPSPH in sensitizing tumors to anti-PD-1 treatment.

Conclusions By tilting the immune balance towards a tumor-friendly composition, PSPH might be useful both as a marker in stratifying patients for ICB therapy, and as an attractive therapeutic target in the treatment of human HCC.

INTRODUCTION

Folate metabolism, which supports a broad set of transformations known as one-carbon (1C) metabolism, is a universal metabolic

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Immune checkpoint blockade (ICB) therapy has a low response rate in the treatment of hepatocellular carcinoma (HCC).

WHAT THIS STUDY ADDS

⇒ High expression of phosphoserine phosphatase (PSPH) promotes tumor immune escape by increasing the recruitment of monocytes/macrophages and reducing the infiltration of CD8⁺ T lymphocytes, attenuating tumor response to immune checkpoint therapy.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ PSPH might be useful both as a marker in stratifying patients for ICB therapy and as an attractive therapeutic target in the treatment of HCC.

process that serves to activate and transfer 1C units for biosynthetic processes.^{1–3} Its products include purine, thymidine, methionine, nicotinamide adenine dinucleotide phosphate (NADPH), and so on, and primarily impact DNA synthesis, epigenetics and mitochondrial redox homeostasis.^{4–8} 1C metabolism has been found upregulated in many tumors and targeting 1C metabolism has led to the first selective chemotherapeutic agents (methotrexate) in the treatment of leukemias.^{9–11} New genomics and metabolomics approaches have highlighted distinctive aspects of 1C metabolism in cancer and rekindled interest in targeting this pathway with more selective modulators. However, studies about tumor 1C metabolism have been largely focused on cancer cells, with its possible impact on or interaction with tumor immune environments largely unknown. Moreover, given the complexity of 1C metabolism, the lack of an effective single target for 1C metabolism intervention and the limited

inhibitors or modulators currently available for selectively targeting IC enzymes or their products also add to the difficulties of exploiting this pathway for clinical use.

Immune microenvironments influence and determine the clinical outcome and therapeutic responsiveness to immune checkpoint blockade (ICB) treatment of patients with cancer, and a 'cold' tumor microenvironment (particularly low effective T cell infiltration due to physical barrier or an imbalance of respective chemokines, such as high myeloid-related C-C motif chemokine 2 (CCL2) and low CD8-related C-X-C Motif Chemokine 9 (CXCL9) and C-X-C Motif Chemokine 10 (CXCL10) levels) has been indicated as an important biomarker for resistance to ICB treatment for some tumors.^{12–17} However, mechanisms involved in regulating the balance of specific chemokine productions in tumor environments have not been fully understood. Deciphering mechanisms governing chemokines balance might assist physicians in distinguishing patients who are most likely to benefit from ICB therapy.

In the current study, we aimed to figure out possible changes in IC metabolism in human hepatocellular carcinoma (HCC), its relationship with tumor immune environments and how their possible interaction might influence the responsiveness of tumor to ICB therapeutics. Our results showed that an upstream IC pathway enzyme, namely phosphoserine phosphatase (PSPH), was significantly upregulated in tumor than non-tumor liver tissues of human HCC, and its level exhibited a positive correlation with disease progression. While it did not show direct effects on the physiology of cancer cells themselves, PSPH in cancer cells could induce monocytes/macrophages infiltration and reduce CD8⁺ T cells recruitment into the tumor milieu through increasing CCL2 expression and suppressing CXCL10 production, respectively. PSPH knockdown, alone or in combination with ICB agents, could significantly reduce tumor progression in mice in vivo. Therefore, PSPH might represent itself as both a possible diagnostic landmark and a candidate for immune-based cancer therapies for patients with HCC.

MATERIALS AND METHODS

Human HCC tissue samples

Liver tissue samples were obtained from 351 untreated patients with pathologically confirmed HCC at Sun Yat-sen University Cancer Center and Sun Yat-sen Memorial Hospital between 2006 and 2020. Patients with concurrent autoimmune disease, HIV, or syphilis were excluded. Among these patients, 321 (cohort 1) who had clinical biochemistry test results and follow-up data were used for immunohistochemical staining and subsequent correlations and overall survival (OS) analysis. Another 30 fresh liver tissues (cohort 2) were used for RNA, protein, and metabolites-related experiments. Non-tumor sites were defined as areas at least 3 cm away from tumor sites. The clinical characteristics of all patients are summarized in online supplemental table S1.

Animals

Wild-type male C57BL/6 mice and Balb/c nude mice were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China), and NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22} mice (NCG) mice were purchased from GemPharmatech (Nanjing, China). All mice were maintained under specific pathogen-free conditions and were used between 6 weeks and 8 weeks of age. Mice were also euthanized when they experienced open skin lesions, weight loss >15% total body weight, or failed to thrive.

Mouse tumor models and treatments

Subcutaneous tumor model: A total of 8×10⁵ Hepa1-6, shPSPH-Hepa1-6, and shPLKO-Hepa1-6 cells were subcutaneously transplanted into the flanks of mice. Mice were monitored for tumor growth every 3 days. Tumor size was measured by use of a caliper and calculated using the formula Volume = (length)(width)²/2. The endpoint was defined as the time at which a progressively growing tumor reached 2000 mm³ in volume.

Orthotopic hepatic tumor model: A total of 8×10⁵ tumor cells were suspended in 25 μl of 50% basement membrane extract (3432-005-01, Trevigen), and intrahepatically injected into the left lobe of the liver of anesthetized 6-week-old C57BL/6 mice. Mice bearing luciferase-expressing Hepa1-6 tumors were intraperitoneally injected with 1.5 mg D-luciferin (2779, Biovision) to monitor orthotopic tumor growth with a Xenogen in vivo imaging system (PerkinElmer).

In some experiments, 10 mg/kg GdCl₃ (4399770, Sigma-Aldrich) was administered intraperitoneally every 3 days beginning on day 0 to deplete monocytes and macrophages. In some experiments, Rat IgG2a isotype control antibody (BP0089, Bioxcell), or 100 μg of anti-mouse CD8a antibody (BE0004, Bioxcell) were intraperitoneally injected beginning on day 6, and tumor infiltrated Ly6C⁺ monocytes and F4/80⁺ macrophages were intratumorally injected beginning on day 6. In other experiments, Rat IgG2a isotype control antibody, 50 μg of anti-mouse programmed cell death protein 1 (PD-1) antibody (BE0146, Bioxcell), 2 mg metformin (HY-17471A, MedChemExpress), or 2 mg metformin in combination with anti-mouse PD-1 antibody were intraperitoneally injected beginning on day 6 or day 9 (metformin: every day; antibodies: every 3 days).

Statistical analysis

Statistical tests used are indicated in the figure legends. Data were tested for normality using Shapiro–Wilk test or Kolmogorov–Smirnov test and variance homogeneity using F-test. Correlations between parameters were measured by Pearson correlation. Survival curves were calculated by the Kaplan–Meier method and analyzed by the log-rank test. The Cox proportional hazards model was used to identify prognostic factors through univariate and multivariate analyses. For comparing normally distributed continuous variables that were homogeneity

of variance, we used a two-tailed Student's t-test or two-way analysis of variance with Bonferroni's multiple comparisons test, respectively. For comparing normally distributed continuous variables that were heterogeneity of variance, we used Welch's t-test. The results are expressed as the means±SEMs. Statistical analysis was performed with GraphPad Prism V.9 (GraphPad Software, La Jolla, California, USA). The following are the thresholds for statistical significance: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$; ns, no significance.

Further details of materials and methods are provided in the online supplemental materials.

RESULTS

1C metabolism is upregulated in human HCC and levels of PSPH expression are positively correlated with disease progression

Transcriptome data from The Cancer Genome Atlas and fresh HCC tissues (50 and 5 patients, respectively, with paired tumor and non-tumor data) showed that levels of 1C metabolic enzymes expression were significantly different between tumor and paired non-tumor liver tissues, with *PSPH*, *methionine adenosyltransferase 2A (MAT2A)*, *methylentetrahydrofolate dehydrogenase 1 like (MTHFD1L)*, *aldehyde dehydrogenase 1 family member L2 (ALDH1L2)*, *serine hydroxymethyltransferase 2 (SHMT2)*, *phosphoglycerate dehydrogenase (PHGDH)* increased, and *aldehyde dehydrogenase 1 family member L1 (ALDH1L1)*, *methionine adenosyltransferase 1A (MAT1A)* decreased in tumors (figure 1A–C). Western blotting confirmed the difference in PSPH, MAT2A, MTHFD1L, ALDH1L2, ALDH1L1 and MAT1A expression between paired tumor and non-tumor tissues (figure 1D and online supplemental figure S1A, $n=3$). Consistently, intermediate metabolites from the main 1C pathway—3-phosphoserine (3Pser), serine, and methionine—were higher, while glycine from the branch 1C pathway was lower in tumor than paired non-tumor tissues (figure 1E,F, $n=5$).

PSPH was the most significantly changed 1C enzyme in HCC tumors. While exhibiting very low levels of expression in non-tumor liver tissues, PSPH was increased in tumors as validated through quantitative PCR (Q-PCR), western blotting, and immunohistochemistry (IHC) staining analysis (figure 1G–I, $n=17$ for Q-PCR, $n=7$ for western blotting and IHC). When patients with HCC who had received curative resection with follow-up data were divided into two groups according to the median value of their PSPH expression level in tumor tissues, as shown in figure 1J, PSPH expression was found negatively associated with the OS of patients with HCC (PSPH^{high}: $n=160$, PSPH^{low}: $n=161$, $p=0.001$). Moreover, levels of PSPH expression in tumor tissues were positively associated with aspartate aminotransferase/alanine aminotransferase (AST/ALT), P⁺, and lactate dehydrogenase (LDH) levels in the peripheral blood of paired patients with HCC (online supplemental figure S2), and could serve as an independent prognostic factor for the OS of patients with

HCC ($p=0.027$, online supplemental table S2). These results suggested that 1C metabolism was switched on in human HCC tissues, and its key enzyme—PSPH—might be upregulated to facilitate disease progression.

PSPH orchestrates macrophages and CD8⁺ T cells accumulation in tumor microenvironments and promotes tumor growth in mice in vivo

To understand the protumor effects of PSPH, two sh*PSPH* RNA sequences (designated as sh*PSPH1* and sh*PSPH2*) were designed to transfect Hepa1-6 tumor cells. While neither sh*PSPH* RNA impacted tumor cell viability and apoptosis in vitro, they could both significantly inhibit tumor growth in immunocompetent C57BL/6 mice in vivo compared with the sh*PLKO* control (empty cloning vector) (online supplemental figure S3, $n=4$ and figure 2A, $n=5$). In contrast, no significant difference in terms of tumor growth was displayed between the sh*PSPH*-Hepa1-6 and sh*PLKO*-Hepa1-6 tumors when they were transplanted into the NCG mice (figure 2B, $n=6$), and only a modest difference was observed in monocytes/macrophages-depleted mice (figure 2C, $n=3$), or Balb/c nude mice (figure 2D, $n=3$), suggesting that the pro-tumor effects of PSPH might be mediated through both macrophages and T lymphocytes. Supporting this hypothesis, replenishment of tumor-associated monocytes and macrophages, or depletion of CD8⁺ T cells, or a combination of tumor-associated monocytes and macrophages replenishment with CD8⁺ T cells depletion could effectively relieve the attenuated tumor growth of sh*PSPH*-Hepa1-6 cells in comparison to the sh*PLKO* control in C57BL/6 mice in vivo (online supplemental figure S4, $n=4$).

To explore how PSPH-depleted cancer cells might impact immune components, mRNA array was performed to compare genes differently expressed between sh*PSPH* and sh*PLKO* hepatoma cells (PLC/PRF/5 and SNU449 cells; online supplemental figure S5A). Of the enriched pathways, immune cell chemotaxis-related genes, such as *CCL2* and *CXCL10*, were substantially downregulated or upregulated in sh*PSPH* cells compared with the sh*PLKO* controls, while major histocompatibility complex (MHC) class genes, such as *humanleukocyteantigen A (HLA-A)* and *humanleukocyteantigen B (HLA-B)*, were markedly increased (online supplemental figure S5B,C), implicating PSPH might impact immune infiltration, as well as T cell activation through chemokines and MHC-related pathways. To test the chemokine hypothesis, we analyzed the levels of monocytes/macrophages-related *CCL2* and CD8⁺ T cells-related *CXCL10* in our in vivo mice models. Results showed that levels of *CCL2* expression were significantly reduced while levels of *CXCL10* were markedly increased in sh*PSPH*-transfected Hepa1-6 tumors than in the sh*PLKO* controls in C57BL/6 mice (figure 2E, $n=5$). Accordingly, when measured by flow cytometry, levels of monocytes (Ly6C⁺ cells) and macrophages (F4/80⁺ cells) infiltration were reduced in both sh*PSPH1*-transfected and sh*PSPH2*-transfected tumors, while levels of CD8⁺

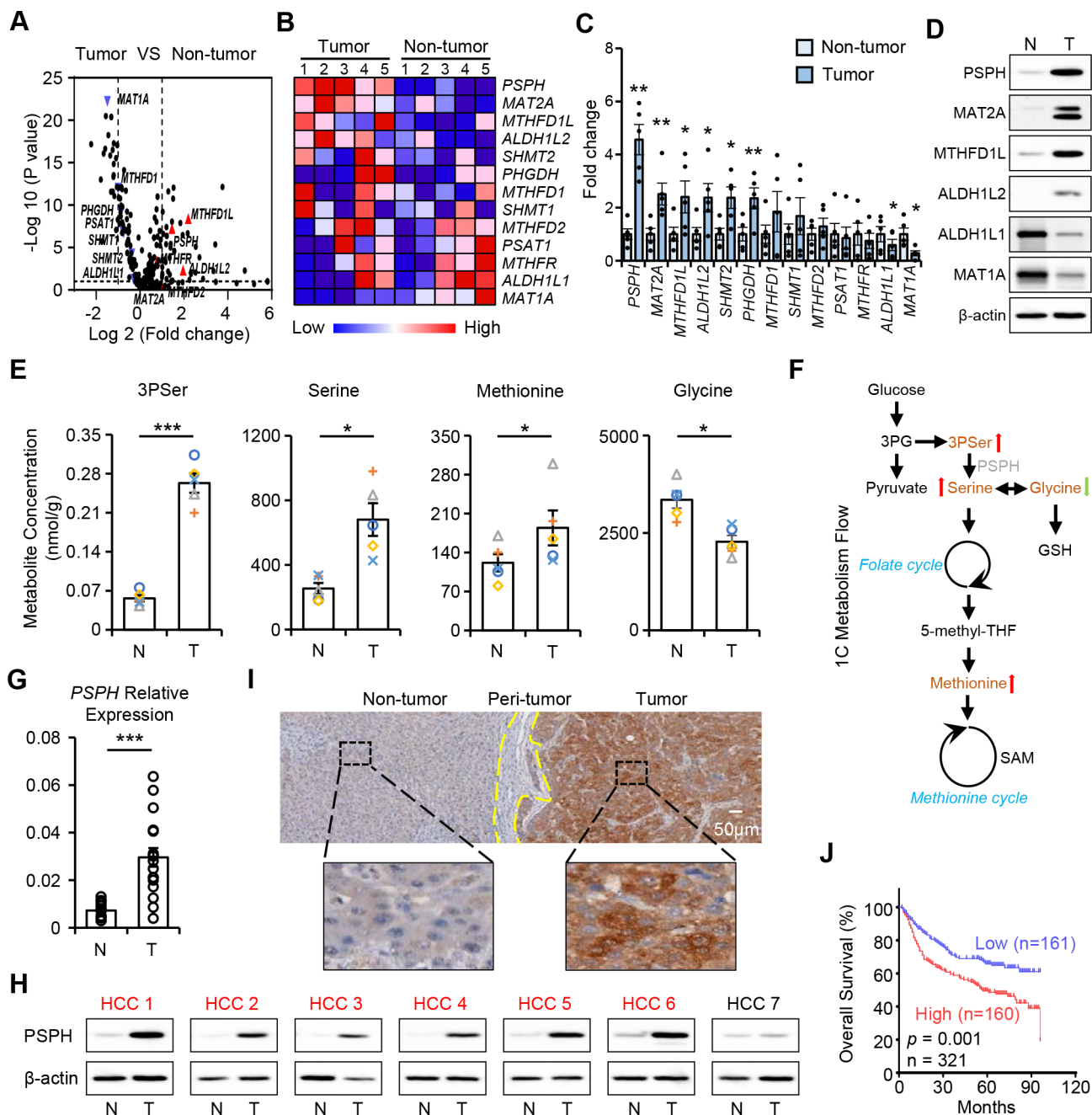


Figure 1 One-carbon (1C) metabolism is upregulated in human hepatocellular carcinoma (HCC) and levels of phosphoserine phosphatase (PSPH) expression are positively correlated with disease progression. (A–C) Transcriptome data from The Cancer Genome Atlas ($n=50$, (A)) and fresh HCC tissues ($n=5$, (B–C)). (D) 1C enzymes in fresh HCC tissues were determined by western blotting ($n=3$; N: non-tumor; T: tumor). (E–F) Key 1C metabolites in fresh HCC tissues were analyzed by mass spectrometry ($n=5$; N: non-tumor; T: tumor). (G–I) PSPH expression in fresh HCC tissues were determined by quantitative PCR (Q-PCR) ($n=17$, (G)), western blotting ($n=7$, (H)) and IHC staining ($n=7$, (I)) (N: non-tumor; T: tumor). (J) Overall survival of patients with HCC with high or low tumor expression of PSPH. Data are mean \pm SEM. Statistical methods: paired t-test (A, C, E), Welch's t-test (G), Kaplan-Meier method and log-rank test (J). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. 3PG, 3-phosphoglycerate; GSH, glutathione; SAM, S-adenosyl-methionine.

T cells infiltration were significantly increased in those PSPH-depleted tumors compared with their sh*PLKO* controls (figure 2F, $n=5$). The reduced accumulation of monocytes (Ly6C⁺ cells) and macrophages (F4/80⁺ cells), and increased infiltration of CD8⁺ T cells in PSPH-depleted tumors compared with sh*PLKO* controls were further confirmed through immunofluorescent staining

of tumor sections (figure 2G,H, $n=5$). Therefore, the above data implied that PSPH expression in tumor cells might facilitate tumor progression through both increasing the CCL2-mediated monocytes/macrophages accumulation and reducing the CXCL10-mediated CD8⁺ T cells infiltration.

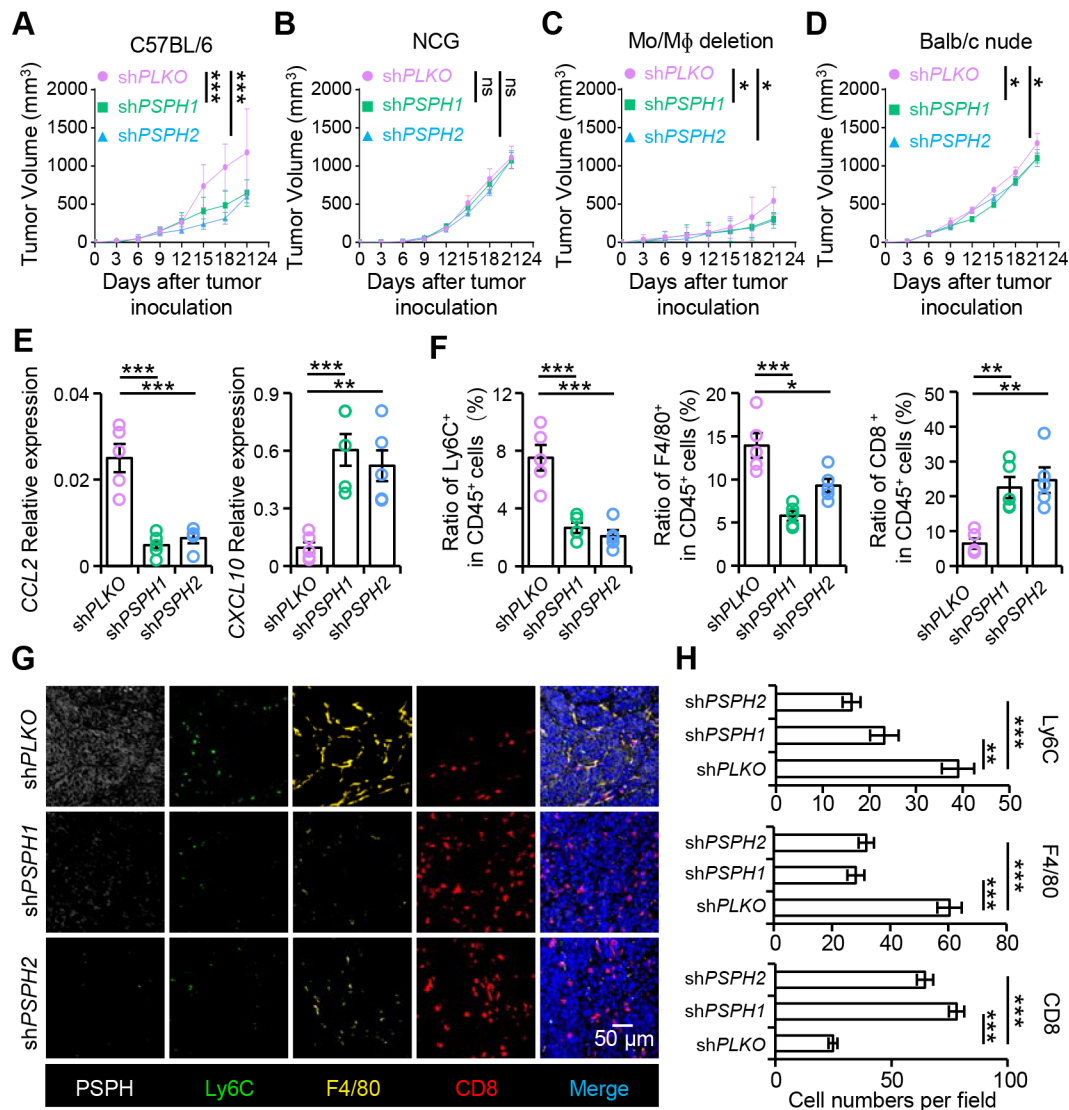


Figure 2 Phosphoserine phosphatase (PSPH) orchestrates macrophages and CD8⁺ T cells accumulation in tumor microenvironments and promotes tumor growth in mice in vivo. (A–D) Tumor growth of shPLKO-transfected or shPSPH-transfected Hepa1-6 cells in C57BL/6 mice (n=5, (A)), NCG (NOD-Prkdc^{em26}Cd52^{Il2rg^{em26}Cd22}) mice (n=6, (B)), monocytes/macrophages-depleted mice (n=3, (C)), or Balb/c nude mice (n=3, (D)). (E) Quantitative PCR (Q-PCR) showed the levels of CCL2 and CXCL10 in shPLKO-transfected or shPSPH-transfected Hepa1-6 tumors in C57BL/6 mice (n=5). (F–H) Flow cytometry (F) and immunofluorescent analysis (G–H) showed the levels of Ly6C⁺ monocytes, F4/80⁺ macrophages, and CD8⁺ T cells infiltration in shPLKO-transfected or shPSPH-transfected Hepa1-6 tumors in C57BL/6 mice (n=5). Data are mean±SEM. Statistical methods: Two-way analysis of variance with Bonferroni's multiple comparisons test (A, B, C, D), Student's t-test (E, F, H). *p<0.05, **p<0.01, ***p<0.001; ns, no significance.

PSPH regulates tumor release of CXCL10 and CCL2 through the S-adenosyl-methionine and glutathione pathways, respectively

To explore mechanisms mediating the tumor release of CCL2 and CXCL10, hepatoma cell lines PLC/PRF/5 and SNU449, which exhibited high basal levels of PSPH expression, were employed in the in vitro experimental models. Interestingly, both PLC/PRF/5 and SNU449 cells expressed very low levels of CCL2 and CXCL10 unless they were stimulated with a low dose of TNF- α . Effects of other cytokines were relatively minor in terms of inducing CCL2 and CXCL10 expression in these cells (online supplemental figure S6A,B, n=3). Since low levels

of TNF- α were prevalent in tumor microenvironments and might be further elevated on various immune therapies, and given that shPSPH1 and shPSPH2 could reduce CCL2 expression while increasing CXCL10 expression compared with shPLKO controls in TNF- α -exposed PLC/PRF/5 and SNU449 cells (figure 3A and online supplemental figure S7A, n=5), we considered this a clinically relevant model to conduct the mechanistic experiments.

It has been reported that CXCL10 expression was negatively regulated by H3K27me₃,¹⁸ whose methylation depended on the methyl groups provided by S-adenosyl-methionine (SAM) downstream of 1C metabolism. Therefore, we first evaluated whether PSPH regulated

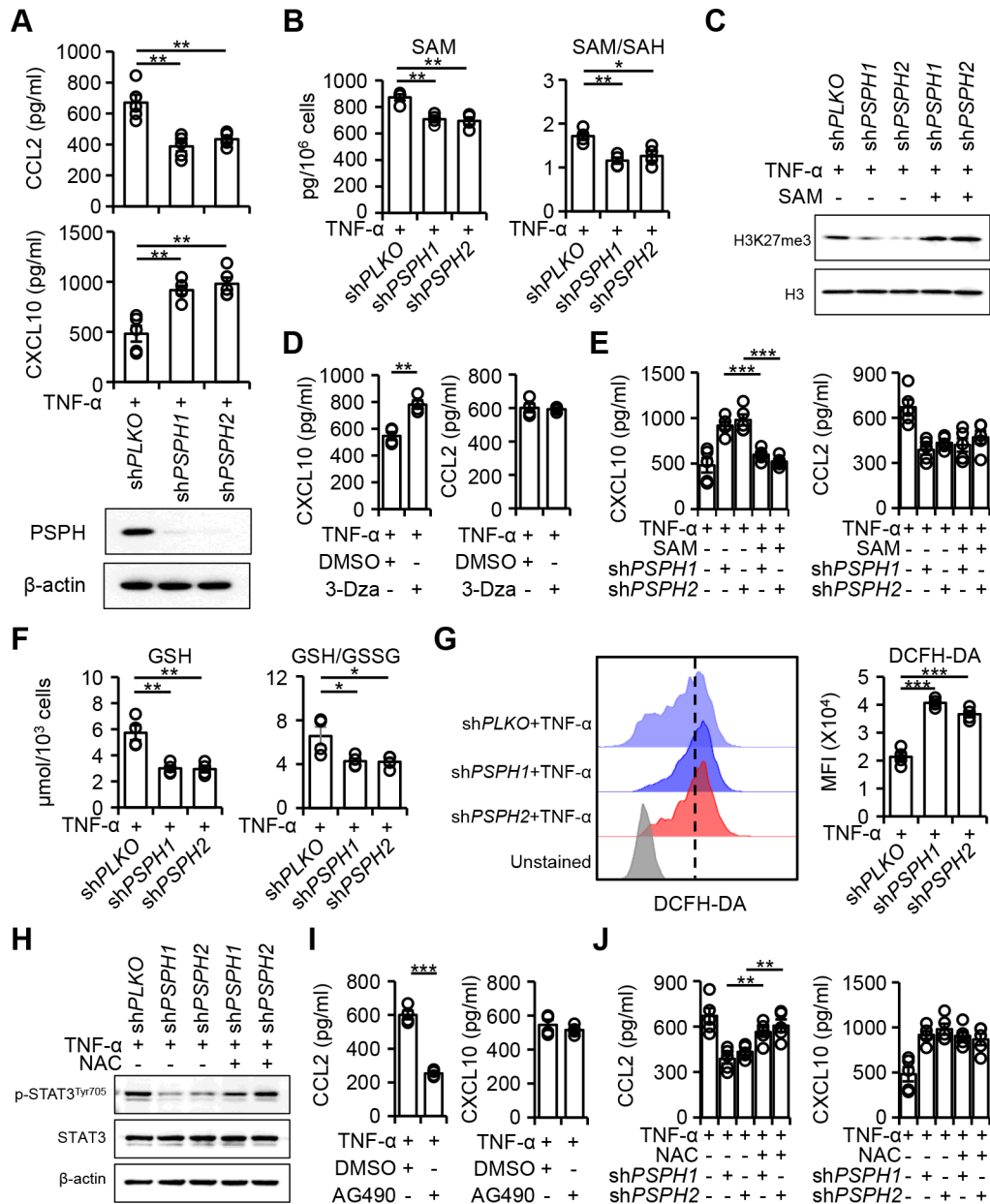


Figure 3 Phosphoserine phosphatase (PSPH) regulates tumor release of CXCL10 and CCL2 through the S-adenosyl-methionine (SAM) and glutathione (GSH) pathways, respectively. PLC/PRF/5 cells were left untreated or transfected with shPLKO or shPSPH, and treated with TNF- α . (A) Western blotting showed PSPH, and ELISA showed CCL2, CXCL10 levels in shPLKO or shPSPH tumor cells (n=5). (B) SAM and SAM/SAH were measured in shPLKO or shPSPH tumor cells (n=4). (C) Western blotting showed H3K27me3 in shPLKO or shPSPH tumor cells, in the presence or absence of supplemented SAM (n=3). (D-E) ELISA showed CCL2 and CXCL10 levels in 3-Deazaadenosine hydrochloride (3-Dza)-treated or 3-Dza-untreated tumor cells (n=4, (D)), or in shPLKO or shPSPH transfected tumor cells, in the presence or absence of supplemented SAM (n=5, (E)). (F) GSH and GSH/GSSG were measured in shPLKO or shPSPH tumor cells (n=4). (G) Flow cytometry analysis showed ROS levels in shPLKO or shPSPH tumor cells (n=4). (H) Western blotting showed p-STAT3^{Tyr705}, STAT3 in shPLKO or shPSPH tumor cells, in the presence or absence of supplemented NAC (n=3). (I-J) ELISA showed CCL2 and CXCL10 levels in AG490-treated or AG490-untreated tumor cells (n=4, (I)), or in shPLKO or shPSPH transfected tumor cells, in the presence or absence of supplemented NAC (n=5, (J)). Data are mean \pm SEM. Statistical methods: Student's t-test (A, B, D, E, F, G, I, J). *p<0.05, **p<0.01, ***p<0.001. SAM, S-adenosyl-methionine (SAM); SAH, S-adenosyl-homocysteine; GSH, glutathione; GSSG, oxidized glutathione; DMSO, dimethyl sulfoxide.

CXCL10 expression through this pathway. As shown in figure 3B,C, online supplemental figures S1B and S7B,C (n=4 for B, n=3 for C), levels of SAM, SAM/SAH, and H3K27me3 were all significantly reduced in shPSPH-transfected PLC/PRF/5 and SNU449 cells compared

with their respective shPLKO counterparts. Notably, 3-Deazaadenosine hydrochloride (3-Dza), an inhibitor decreasing SAM:SAH ratio and suppressing SAM-dependent methylation reactions, could mimic the effects of shPSPH transfection in increasing CXCL10 expression

in TNF- α -exposed wild-type PLC/PRF/5 and SNU449 cells, while leaving the expression of CCL2 in these cells unimpacted (figure 3D and online supplemental figure S7D, n=4). On the flip side, the addition of SAM could attenuate the increase of CXCL10 expression in shPSPH-transfected PLC/PRF/5 and SNU449 cells compared with their shPLKO controls, while did not impact the expression of CCL2 (figure 3E and online supplemental figure S7E, n=5). Interestingly, shPSPH also increased levels of HLA-A and HLA-B expression in PLC/PRF/5 and SNU449 cells compared with shPLKO controls (online supplemental figure S8A, n=3). While 3-Dza could mimic the effects of shPSPH transfection in increasing both the HLA-A and HLA-B expression in wild-type PLC/PRF/5 and SNU449 cells, the addition of SAM could attenuate the increase of HLA-A and HLA-B expression in shPSPH-transfected PLC/PRF/5 and SNU449 cells compared with their shPLKO controls (online supplemental figure S8A,B, n=3). These data suggested that PSPH-mediated high levels of SAM and H3K27me3 might inhibit the expression of CXCL10 and HLA-A/B by hepatoma cells, and depletion of PSPH could release the production or expression of these molecules.

Glutathione (GSH) is another essential downstream metabolite of the IC pathway that can interact with reactive oxygen species (ROS) to regulate cellular redox balance.¹⁹ As shown in figure 3F,G and online supplemental figure S7F,G (n=4), shPSPH-transfected PLC/PRF/5 and SNU449 cells reduced GSH level and GSH/GSSG ratio, while increasing the ROS level in comparison to their shPLKO counterparts. Given that ROS could regulate several signaling pathways and their respective downstream functions, we tried to explore which pathway might be impacted by shPSPH transfection and possibly mediate CCL2 expression by hepatoma cells. Gene enrichment analysis of the transcriptome profiles of shPSPH-transfected hepatoma cells or shPLKO-transfected control cells showed that the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway was preferentially inhibited in shPSPH-transfected hepatoma cells (p=0.049 for PLC/PRF/5 and p=0.010 for SNU449), with other signaling pathways such as TNF- α , mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase-protein kinase B (PI3K-AKT) largely unimpacted (online supplemental figure S9, n=2). Interestingly, the protein levels of Phospho-Stat3 (Ser705) (p-STAT3^{Tyr705}), but not other STAT family members, were significantly downregulated in shPSPH-transfected PLC/PRF/5 and SNU449 cells compared with shPLKO controls (figure 3H, online supplemental figures S1C and S7H, n=3 and data not shown). Importantly, N-Acetyl-L-cysteine (NAC, a ROS scavenger) could antagonize the effects of shPSPH to re-induce the expression of p-STAT3^{Tyr705} in shPSPH-transfected PLC/PRF/5 and SNU449 cells (figure 3H and online supplemental figure S7H, n=3), implying that the effect of PSPH on signal transducer and activator of transcription

3 (STAT3) activation was mainly mediated through ROS. When chemokines were measured, AG-490, a STAT3 signaling inhibitor, could mimic the effects of shPSPH in reducing the production of CCL2 in wild-type PLC/PRF/5 and SNU449 cells, while leaving those of CXCL10 unimpacted (figure 3I and online supplemental figure S7I, n=4), and NAC, which re-induced p-STAT3^{Tyr705} in shPSPH-transfected hepatoma cells, could abrogate the shPSPH-induced inhibition of CCL2 production in these cells compared with their shPLKO controls, while it did not impact the expression of CXCL10 (figure 3J and online supplemental figure S7J, n=5). Together, these data indicated that PSPH-mediated ROS scavenging by GSH might induce CCL2 production in hepatoma cells through the activation of the STAT3 signaling pathway.

We also compared the difference between TNF- α -untreated and TNF- α -treated hepatoma cells in terms of their levels of the above-mentioned pathways. As shown in online supplemental figure S6C,D, TNF- α did not impact ratios of SAM/SAH, but could decrease levels of H3K27me3 in cancer cells, which explained its effects in modestly inducing CXCL10 production by these cells (online supplemental figure S6A). TNF- α did not impact levels of GSH/GSSG and ROS expression in cancer cells (online supplemental figure S6E,F), and the basal levels of p-STAT3^{Tyr705} in untreated cells were relatively low (online supplemental figure S6G), possibly due to a lack of stimulation. When cells were stimulated with TNF- α , they substantially increased levels of p-STAT3^{Tyr705} (online supplemental figure S6G) and thus upregulated the production of CCL2 (online supplemental figure S6B). The TNF- α -induced upregulation of CXCL10 and CCL2 could be significantly enhanced or abrogated by treating these cells with shPSPH (figure 3A).

Correlations between the PSPH level, p-STAT3/H3K27me3 level, CCL2/CXCL10 expression, and macrophages/effector T cells infiltration in human HCC samples

To confirm the above findings concerning mechanisms regulating CCL2 and CXCL10 expression by PSPH, serial sections from 20 patients with HCC were stained with different markers. As shown in figure 4A–C, levels of PSPH were found positively associated with those of p-STAT3, CCL2, and CD68⁺ cells infiltration in tumor tissues of HCC (PSPH and p-STAT3: r=0.6202, p=0.0035; PSPH and CCL2: r=0.7217, p=0.0003; PSPH and CD68⁺ cells: r=0.6963, p=0.0006), and while they were positively associated with levels of H3K27me3 expression (r=0.6555, p=0.0017), levels of PSPH were negatively correlated with those of CXCL10 and CD8⁺ T cells infiltration in HCC tumors (PSPH and CXCL10: r=-0.6206, p=0.0035; PSPH and CD8⁺ T cells: r=-0.6205, p=0.0035). Consistently, levels of p-STAT3 expression were positively correlated with CCL2 expression and CD68⁺ cells infiltration (figure 4A,D; p-STAT3 and CCL2: r=0.7493, p=0.0001; p-STAT3 and CD68⁺ cells: r=0.6507, p=0.0019), while levels of H3K27me3 expression were negatively associated with CXCL10 expression and CD8⁺ T cells

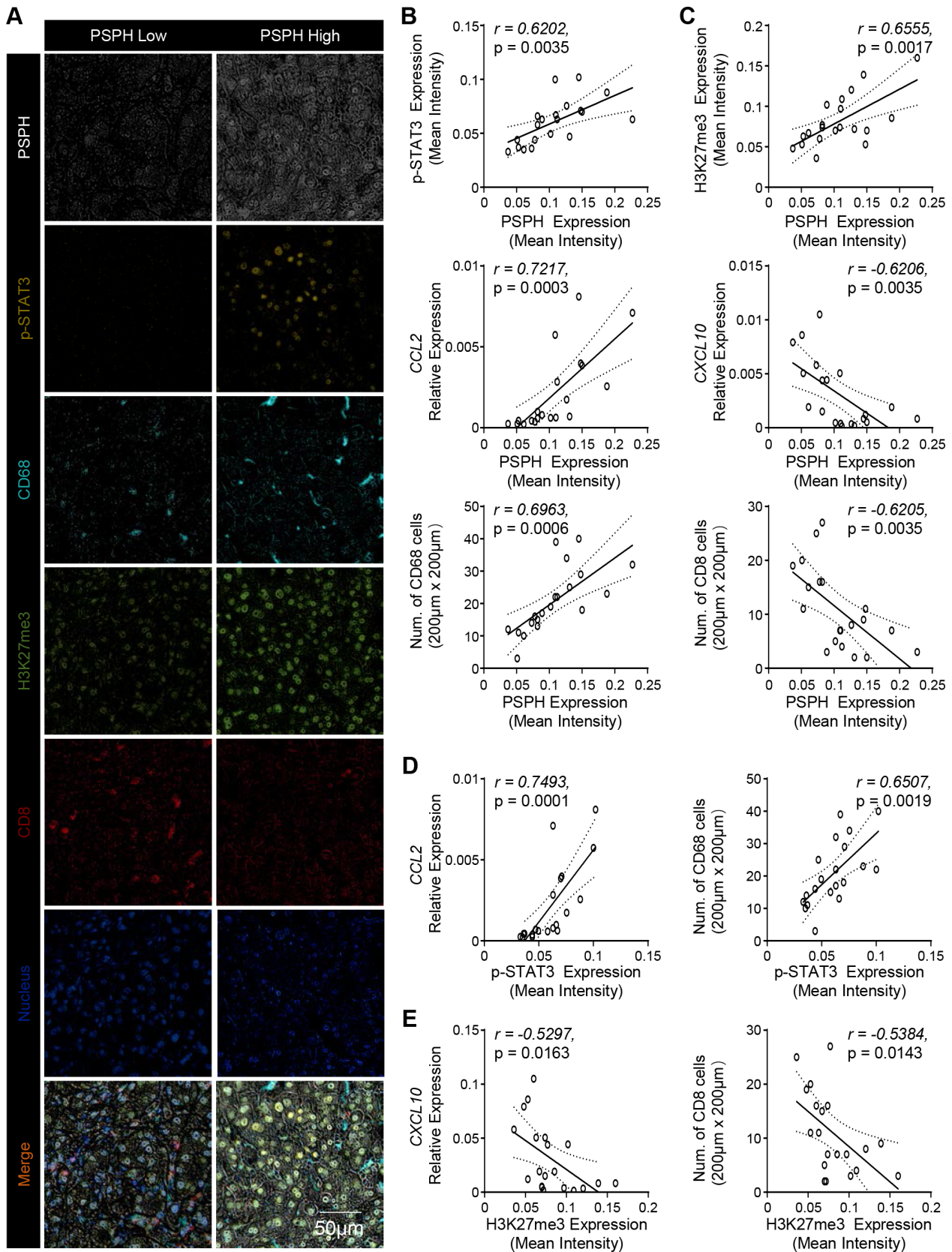


Figure 4 Correlations between the phosphoserine phosphatase (PSPH) level, p-STAT3/H3K27me3 level, *CCL2*/*CXCL10* expression, and macrophages/effector T cells infiltration in human hepatocellular carcinoma (HCC) samples. (A) Immunofluorescent analysis showed the expression of indicated markers in HCC tumor sections with low or high levels of PSPH. (B–E), Correlation between the levels of PSPH expression, p-STAT3 expression, H3K27me3 expression, *CCL2* mRNA, *CXCL10* mRNA, CD68⁺ cells infiltration, and CD8⁺ T cells infiltration in HCC tumor samples were analyzed by quantitative PCR (Q-PCR) or IHC. n=20. Statistical methods: Pearson correlation and linear regression analysis (B, C, D, E).

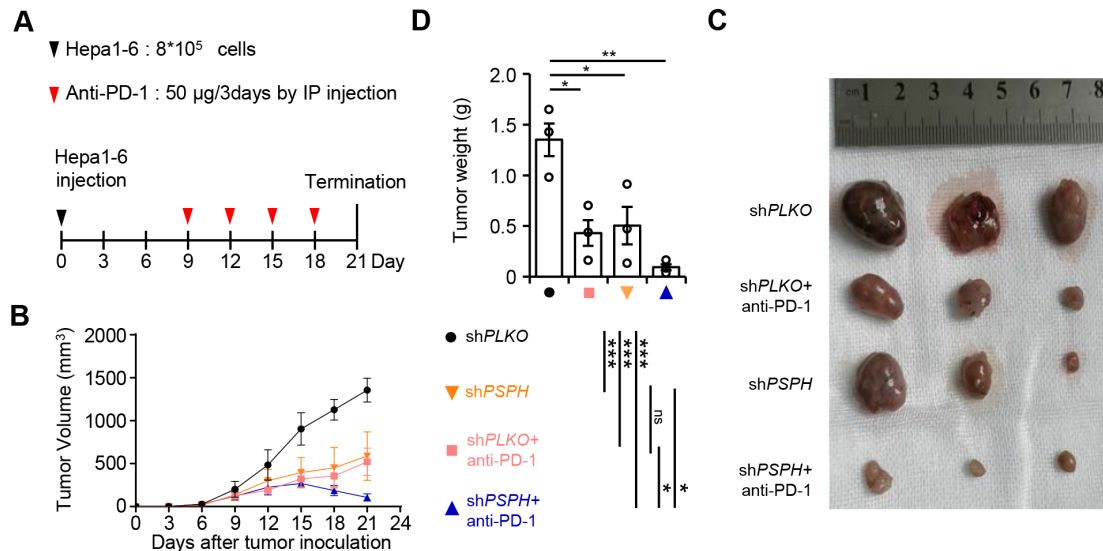


Figure 5 shPSPH sensitizes hepatoma to anti-PD-1 antibody treatment in subcutaneous mice model. (A) C57BL/6 mice with established shPLKO-transfected or shPSPH-transfected Hepa1-6 tumors were intraperitoneally injected with or without anti-PD-1 antibodies at indicated times. (B–D) Tumor growth (B, C) (day 21), and tumor weight (D) (day 21) were monitored. $n=3$. Data are mean \pm SEM. Statistical methods: Two-way analysis of variance with Bonferroni's multiple comparisons test (B), Student's t test (D). * $p<0.05$, ** $p<0.01$, *** $p<0.001$; ns, no significance. IP, intraperitoneally injected.

infiltration in tumors of HCC (figure 4A,E; H3K27me3 and *CXCL10*: $r=-0.5297$, $p=0.0163$; H3K27me3 and CD8⁺ T cells: $r=-0.5384$, $p=0.0143$).

shPSPH sensitizes hepatoma to anti-PD-1 antibody treatment in mice in vivo

Tipping the scale towards a quantitatively and qualitatively immune-effective microenvironment is essential to T cell-related immunotherapies. We went on to establish whether the depletion of PSPH expression could synergistically act with ICB therapeutics to inhibit tumor progression of HCC. Wild-type C57BL/6 mice were subcutaneously inoculated with shPLKO-Hepa1-6 cells or shPSPH-Hepa1-6 cells, before being treated with or without anti-PD-1 antibodies (figure 5A). As shown in figure 5B–D ($n=3$), tumor growth was inhibited in the shPSPH-transfected group, or the shPLKO-transfected tumor treated with anti-PD-1, in comparison to the shPLKO control in terms of both tumor volume and tumor weight. Importantly, the combination of PSPH depletion with anti-PD-1 was more effective in prohibiting tumor growth than either treatment alone, indicating a synergistic effect between these two therapeutic treatments.

Orthotopic Hepa1-6 hepatoma mice model was also established to confirm the above observations. Wild-type C57BL/6 mice with orthotopic shPLKO-Hepa1-6 or shPSPH-Hepa1-6 hepatomas were treated with or without anti-PD-1 antibodies (figure 6A). As shown in figure 6B–E ($n=3$), shPSPH-Hepa1-6 hepatomas showed reduced *CCL2* expression, increased *CXCL10* production, reduced Ly6C⁺ cells and F4/80⁺ cells infiltration, and increased CD8⁺ T cells accumulation in comparison to their respective shPLKO controls. Similar to those observed in subcutaneous mice models, tumor growth was inhibited in the shPSPH transfected group, or the shPLKO-transfected

tumor treated with anti-PD-1 antibodies compared with the shPLKO control, while the combination of PSPH depletion with anti-PD-1 was more effective in prohibiting tumor growth than either treatment alone (figure 6F,G), confirming a synergistic effect between these two therapeutic treatments.

Moreover, our results also showed that CD8⁺ T cells purified from shPSPH-Hepa1-6 tumors (both subcutaneous and orthotopic) exhibited higher expression of Granzyme B and perforin in comparison to their shPLKO-tumor derived counterparts—although at levels much lower than those purified from tumors treated with anti-PD-1 antibodies (online supplemental figure S10, $n=3$)—an effect which might be explained by the higher expression of *HLA-A* and *HLA-B* molecules by shPSPH tumor cells as observed in in vitro experiments (online supplemental figure S8A,B, $n=3$).

Metformin mimics the effects of shPSPH in regulating chemokines production, immune composition, and hepatoma growth in mice in vivo

Given that no PSPH inhibitor is currently available and metformin, an antidiabetes and an antiaging chemical, has been implicated to be able to modulate tumor metabolism,^{20–22} we tried to explore whether metformin might impact PSPH expression and thus influence HCC tumor progression. First, our results showed that metformin did not directly impact the viability and apoptosis of Hepa1-6 cells in vitro (online supplemental figure S11A,B, $n=4$), but could reduce levels of PSPH expression in Hepa1-6 tumors in C57BL/6 mice in vivo (figure 7A, $n=4$), while leaving other major enzymes of the IC pathway, such as MTHFD1L, ALDH1L2, and MAT2A largely unimpacted (figure 7A, online supplemental figure S11C, $n=4$). Meanwhile, metformin could reduce *CCL2* expression,

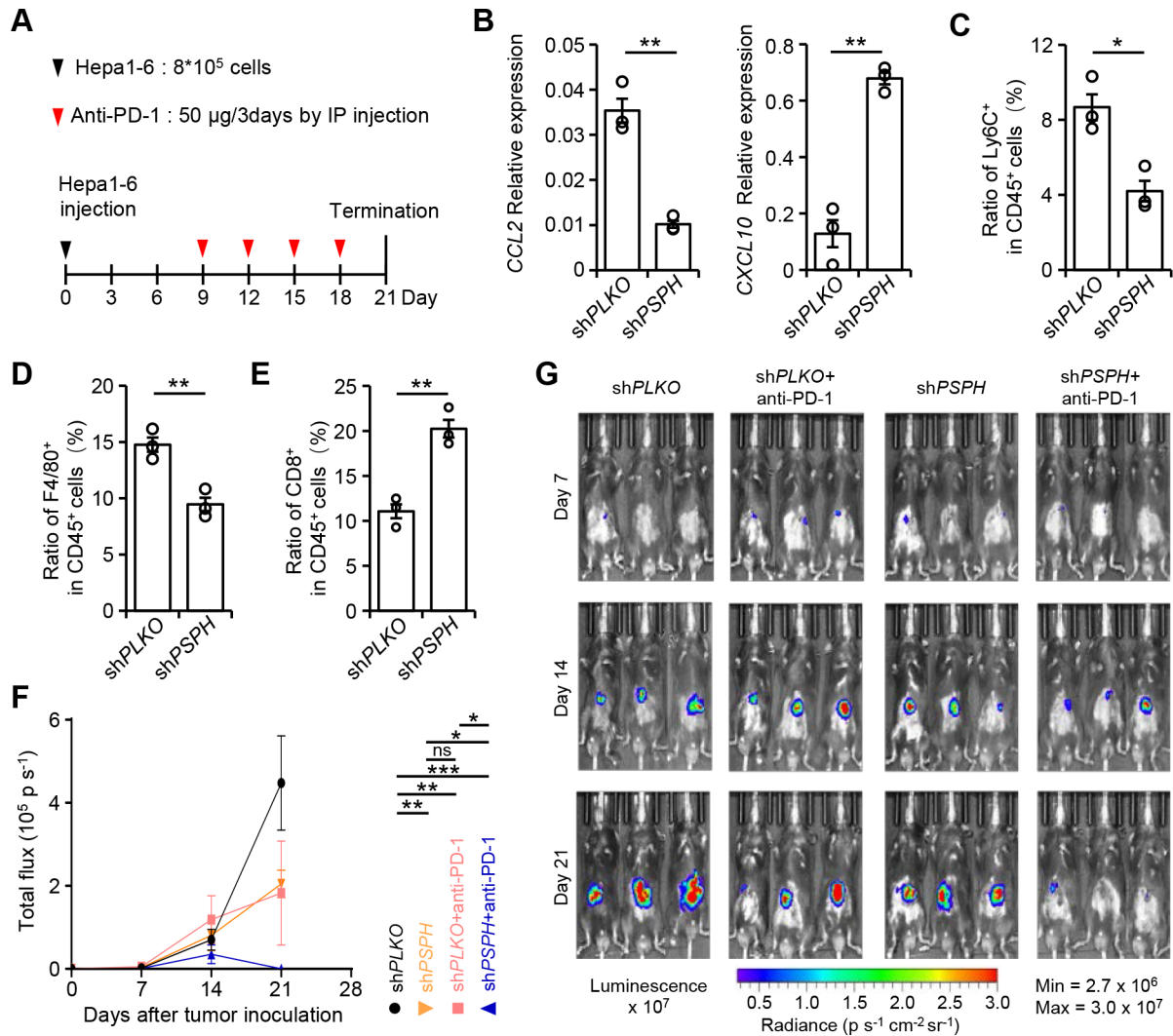


Figure 6 shPSPH sensitizes hepatoma to anti-PD-1 antibody treatment in orthotopic mice model. (A) C57BL/6 mice with established orthotopic shPLKO-transfected or shPSPH-transfected, luminescence-positive, Hepa1-6 tumors were intraperitoneally injected with or without anti-PD-1 antibodies at indicated times. (B–E) *CCL2* and *CXCL10* levels (n=3, (B)), Ly6C⁺ monocytes, F4/80⁺ macrophages, and CD8⁺ T cells infiltrations (n=3, (C–E)) in tumors were determined by quantitative PCR (Q-PCR), and flow cytometry, respectively. (F–G) Real-time tumor growth was monitored. n=3. Data are mean \pm SEM. Statistical methods: Two-way analysis of variance with Bonferroni's multiple comparisons test (F), Student's t-test (B, C, D, E). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, no significance. IP, intraperitoneally injected.

increase *CXCL10* production, reduce Ly6C⁺ cells and F4/80⁺ cells infiltration, and increase CD8⁺ T cells accumulation in tumor tissues compared with their respective control treatment groups, which mirrored the effects of shPSPH transfection of Hepa1-6 tumors (figure 7B–E, online supplemental figure S11D, n=3). Notably, while anti-PD-1 or metformin treatment could inhibit tumor growth both in size and weight compared with the non-treated control in C57BL/6 mice, anti-PD-1 combined with metformin could reduce tumor growth more effectively than either treatment alone (figure 7F–H, n=3), an effect also similar to that observed from anti-PD-1 together with PSPH depletion.

DISCUSSION

The ever-changing immune components—in particular, the intrinsic or developed myeloid-cell-rich and effector-T cell-low tumor microenvironment—poses unique challenges for patients with cancer to mount effective responses to ICB therapy.^{23–26} Unveiling the basic mechanisms determining the immune balance of tumor environments would facilitate the search for biomarkers that could predict clinical responses in different tumor types and for individual patients. Our current study provided evidence that PSPH from the 1C metabolic pathway might play an important yet unexpected role in determining the immune composition of tumor microenvironments of HCC. On cytokine stimulation, PSPH in cancer cells favored the infiltration of myeloid cells via the GSH-CCL2 axis, while prohibiting the infiltration of effector T cells via the SAM-CXCL10 pathway, thus tipping the

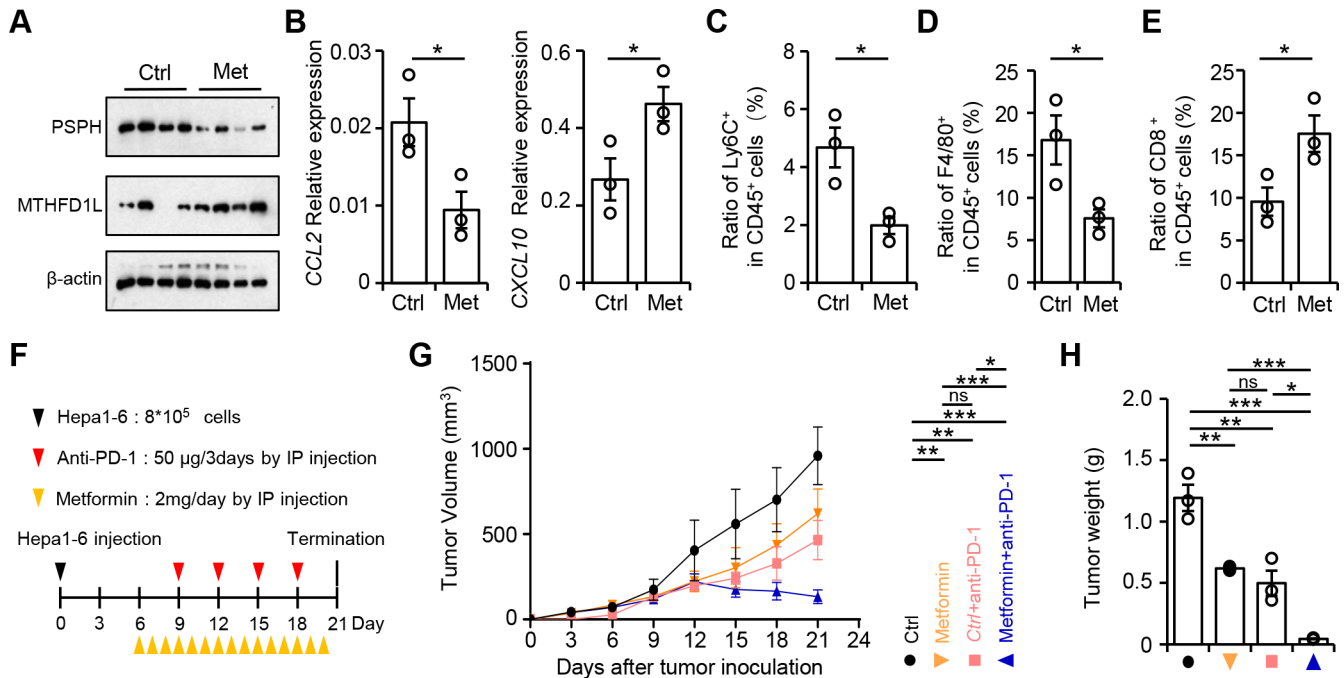


Figure 7 Metformin mimics the effects of shPSPH in regulating chemokines production, immune composition, and hepatoma growth in mice in vivo. (A–E) Effects of Metformin (Met) on PSPH and MTHFD1L expression (n=4, (A)), CCL2 and CXCL10 levels (n=3, (B)), and Ly6C⁺ monocytes, F4/80⁺ macrophages, CD8⁺ T cells infiltration (n=3, (C–E)) in Hepa1-6 tumors of C57BL/6 mice were determined by western blotting, quantitative PCR (Q-PCR), and flow cytometry, respectively. (F–H) C57BL/6 mice with established Hepa1-6 tumors were intraperitoneally injected with or without metformin, anti-PD-1 antibodies at indicated times (F), Tumor growth (n=3 for (G), n=3 for (H) (day 21)) was monitored. Ctrl: PBS. Data are mean \pm SEM. Statistical methods: Student's t-test (B, C, D, E, H), Two-way analysis of variance with Bonferroni's multiple comparisons test (G). *p<0.05, **p<0.01, ***p<0.001; ns, no significance. IP, intraperitoneally injected; PBS, phosphate buffer saline.

scale towards immunosuppression and attenuating tumor responses to ICB therapies.

Tumor microenvironments are heterogeneous in terms of both cell composition and nutrient availability, and some metabolic shifts observed in cancer cells might not be unique to the tumor milieu.^{27–29} Therefore, the prospect of targeting the tumor metabolic pathway is rationally probable but practically challenging. Yet recent studies on metabolism demonstrate that tumors and T cells compete for glucose and methionine and that this competition can affect the functions of tumor-infiltrating lymphocytes (TILs), raising the notion that metabolic biomarkers may be crucial factors in antitumor immune responsiveness.³⁰ IC pathways are upregulated in multiple tumor types, with several key enzymes such as PHGDH, SHMT2, methylenetetrahydrofolate dehydrogenase 2 (MTHFD2), thymidylate synthase (TYMS), and dihydrofolate reductase (DHFR) implicated in regulating cancer cell proliferation and being possible drug targets.^{31–34} However, the role of only a limited number of IC pathway members in tumor, especially human HCC, has been explored and the possible role of cancer cell IC metabolism in modulating local immune environments has rarely been documented. Our current study uncovered a novel role of PSPH from IC metabolism in facilitating HCC progression through engendering a myeloid-cell-high, effector-T cell-low tumor microenvironment. Notably, this enzyme exerted different effects

on the expression of CCL2 and CXCL10 by cancer cells. The downregulation of CXCL10 was mediated by SAM, downstream of the IC pathway, presumably through H3K27me3-related regulation of gene expression, while the upregulation of CCL2 was mediated by GSH, which neutralized ROS and thus released the activation of the STAT3 signaling pathway. Importantly, downregulation of PSPH significantly enhanced the antitumor efficacy of anti-PD-1 therapy in mice in vivo, suggesting it is a biomarker as well as a promising drug target for the future design of combination therapies.

Our results showed that shPSPH did not impact hepatoma cell growth per se, which were different from some previous reports showing that PSPH could directly regulate HCC cancer cell behaviors.^{35, 36} The specific cell lines or nutrient-deprived conditions employed by those studies might partially explain the different results. It is worth noting that our in vitro experiments used the complete medium instead of the serine low or free medium, although limited serine supplement might presumably bear more resemblance to the in vivo nutrient-stressed tumor microenvironment, and serine incomplete medium might compensate for the downregulation of PSPH with regards to its downstream effects.³⁷ While the in vivo tumor microenvironments of HCC might exhibit serine stress to some extent due to demands from DNA synthesis, the highly heterogeneous tumor spatio-environments are hard to simulate, and

given the anticipated antiproliferation effects of disruption of 1C metabolism on cancer cells under serine-depleted conditions, a complete medium might be more suitable in terms of dissecting the effects of 1C pathway on cancer cell proliferation from its possible direct role in regulating chemokine expression by these cells. As to why didn't the serine in the complete medium compensate for the *shPSPH*-induced loss of de novo synthesized serine while observing the downstream functions of PSPH, we hypothesized that while *shPSPH* did regulate CXCL10 and CCL2 through its downstream SAM and GSH, respectively, under stress due to the loss of de novo synthesis of serine, the external serine from the medium might preferentially fuel the supply for DNA synthesis, so the cancer cells can keep their growth as observed, which means that downstream pathways other than DNA synthesis might not be sufficiently compensated by serine from the complete medium.³⁸ But such a hypothesis surely requires further exploration and validation.

The current study provided evidence that on cytokine stimulation, PSPH in cancer cells could increase CCL2 expression while inhibiting their production of CXCL10, thus presumably tilting the balance of immune composition towards a myeloid-biased direction in HCC tumor microenvironments, leading to disease progression or ICB resistance.^{23–39} Such finding was consistent with recent reports that a myeloid-biased immune signature was associated with a shift in the myeloid response balance from antitumor to pro-tumor activities, accompanied by enhanced CD8⁺ T cell exhaustion patterns, and effectively predicted recurrence and survival of patients with HCC.⁴⁰ We hypothesize that levels of PSPH expression might therefore serve as a novel biomarker for stratifying patients with HCC for ICB therapy, although the cut-off might need to be adjusted according to different patient cohorts. About 80% of the patients enrolled in the current study exhibited a relative increase of PSPH expression in tumor than in respective non-tumor liver tissue, which might partially explain, if the hypothesis holds true by and large, the relatively low responsiveness to ICB therapy of patients with HCC. Moreover, levels of PSPH not only correlated with those of chemokines expression and myeloid/effector T cells infiltration in HCC tumor tissue, but also associated with AST/ALT, P⁺, and LDH in peripheral blood of the same patients, indicating that, if a correlation model could be further established, levels of PSPH expression in tumor tissue might be able to be translated into/predicted by a combination of peripheral signatures of patients with HCC, which might be of more practical value in terms of clinical usage.

In addition to biomarker, PSPH could also serve as a direct target for immunotherapy in combination with ICB agents. *shPSPH* could lower levels of CCL2 expression and macrophages infiltration while increasing levels of CXCL10 expression and CD8⁺ T cells infiltration. Such shift from myeloid-biased to effector T cell-rich composition in tumor microenvironments might prime the tumor for a better response to immune therapy, and

accordingly, *shPSPH* did sensitize hepatoma to the treatment of anti-PD-1 antibodies in mice in vivo. Notably, no commercial inhibitor selectively targeting PSPH is available currently, so we went a step further to try to figure out whether some currently approved drugs with broad targets might potentially affect the expression of PSPH in cancer cells, and thus represent alternatives to PSPH inhibitors. Interestingly, our results showed that metformin, an antidiabetes and antiaging drug without direct effect on hepatoma cells, could mimic the effects of *shPSPH* by inhibiting PSPH expression in cancer cells, downregulating CCL2 levels and macrophages infiltration, upregulating CXCL10 levels and CD8⁺ T cells infiltration in tumor tissues, and subsequently inhibiting tumor growth in mice in vivo. Importantly, a combination of metformin and anti-PD-1 antibodies exhibited better antitumor effects compared with either treatment alone, representing another similarity between the effects of metformin and *shPSPH*. Of course, ultimately, metformin might or might not act through PSPH to inhibit tumor growth, and the several effects of metformin observed might just be correlations instead of cause-effect relationships. However, for metformin, our current study provided a piece of evidence for its potential repurposed use in the treatment of human HCC, and for PSPH, the effects of metformin arguably provided another layer of evidence for its role in modulating HCC tumor immune environments. Of note, different from a previous study finding that metformin had effects on both energy intake and energy expenditure that were dependent on growth differentiation factor 15 (GDF15),⁴¹ no significant difference between the *shPLKO*-Hepa1-6 and *shPSPH*-Hepa1-6 tumors in terms of their GDF15 levels were found in the current study (data not shown), indicating that metformin might function via distinct mechanisms in different experimental/clinical models.

There are several unsolved problems in the current study. For example, it is unclear what factors might be responsible for determining levels of PSPH expression by HCC cancer cells—possible clues include the availability of extracellular serine or other nutrition-related signaling pathways shifts. As mentioned above, possible mechanisms mediating the effects of metformin on PSPH expression and tumor progression also warrant further exploration. Nevertheless, our current study unveiled a novel and interesting role of PSPH, a member of the 1C metabolism pathway, in modulating immune composition—specifically, regulating the production of CCL2 and CXCL10, and influencing the subsequent balance of myeloid and effector T cells infiltration—in HCC tumor microenvironments. PSPH thus represented a biomarker as well as a potential target for combined therapy with ICB agents in the treatment of HCC.

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Contributors Z-PP designed the experiments, processed tissues, performed flow cytometry/real-time PCR/mouse experiments, collected data, and participated in the writing of the paper. X-CL performed immunohistochemical, immunofluorescence staining, and mouse experiments. Y-HR, DJ and A-QH performed ELISA and western blotting. W-RN and Z-ZJ helped and performed GO/GSEA analysis. LZ and YW planned and supported the project, analyzed data, and wrote the paper. YW was responsible for the overall content as the guarantor.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants. All samples were anonymously coded in accordance with local ethical guidelines (as stipulated by the Declaration of Helsinki). The study protocol was approved by the Institutional Review Board of Sun Yat-Sen University Cancer Center (GZR2020-199). Written informed consent was obtained from each patient before taking part in the study.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository. All data relevant to the study are included in the article or uploaded as supplementary information. The publicly available microarray data sets analyzed in this study from The Cancer Genome Atlas (TCGA) were downloaded from the data portal of Genomic Data Commons (GDC, <https://portal.gdc.cancer.gov/>). The sequencing data have been deposited at the Gene Expression Omnibus with access number GSE193329. The other data generated in this study are included in the article or uploaded as supplementary materials.

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ORCID iDs

Limin Zheng <http://orcid.org/0000-0002-8281-1450>

Yan Wu <http://orcid.org/0000-0002-9022-9794>

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