

USP25 Promotes Glycolysis of Acute Myeloid Leukemia Cells and Enhances Tumor Immunity

Nianxue Wang¹ · Can Yang² · Ruya Zhang¹ · Wei Ren¹ · Biao Shen³✉

Abstract

Acute myeloid leukemia (AML) is an incursionary hematopoietic tumor with frequent relapses and an unfavorable prognosis. Ubiquitin-specific peptidase 25 (USP25) mediates the progression of various cancers, but its role in AML remains uncharted. The expression of USP25 in AML was analyzed *in silico*, *in vitro*, and *in vivo* through real-time quantitative polymerase chain reaction (RT-qPCR) and Western blot. The function of USP25 in proliferation, apoptosis, glycolysis, and immunity in AML was addressed through cell counting kit-8 (CCK-8), flow cytometry, detection of glucose consumption, lactate production, extracellular acidification rate (ECAR), and oxygen consumption rate (OCR), immunohistochemistry, immunofluorescence, and Western blot. The mechanism of USP25 in AML was explored via co-immunoprecipitation (Co-IP), ubiquitination, cycloheximide, and Western blot. Upregulation of USP25 indicated a poor overall survival in AML patients. Knock-in and knock-down results revealed that USP25 enhanced viability, the level of glucose consumption, lactate production, ECAR, and the level of anti-glucose transporter type 1 (GLUT1) and hexokinase-2 (HK2), but reduced the apoptosis rate and the OCR level. Mechanically, USP25 directly bound and deubiquitinated c-Myc. An upregulation of c-Myc and programmed cell death ligand 1 (PD-L1) was found in AML. Knockdown of c-Myc decreased USP25-induced cell viability, glucose consumption, and lactate production level. *In vivo*, knockdown of USP25 decreased tumor volume and weight, the ki-67 expression, and the levels of c-Myc and PD-L1, but elevated the levels of cluster of differentiation 4 (CD4) and cluster of differentiation 8 (CD8). USP25 facilitated proliferation, glycolysis, and immunity through deubiquitinating c-Myc in AML. The results identified USP25 as a possible target for AML treatment.

Keywords

Acute myeloid leukemia · USP25 · Glycolysis · PD-L1 · Immunity · Ubiquitination

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1. Introduction

Leukemia is a treacherous hematologic malignancy. In males and females, the morbidity of leukemia is estimated to rank the ninth and tenth with the 35,670 (4%) and 23,940 (3%) cases, while the mortality ranks the sixth and eighth with the 13,900 (4%) and 9810 (3%) cases in the United States in 2023, respectively (Siegel et al. 2023). Acute myeloid leukemia (AML), featured by aberrant proliferation and differentiation of myeloid progenitors within the bone marrow, is the most prominent leukemia in young adults aged 19–39 years (Miller et al. 2020; Shimony et al. 2025). Multiple treatment approaches, such as chemotherapy, antibody-drug conjugate, radiotherapy, targeted therapy drugs, and hematopoietic stem cell transplantation (Yang and Wang 2018; Miller et al. 2022), have immensely

ameliorated the outcomes of AML patients. Thus, complete remission is achieved in many sufferers with 40%–60% in patients older than 60 years and 60%–85% in those aged 60 years or younger (Miller et al. 2022). However, one of the biggest problems is that about one-half of the patients with complete remission relapse (Döhner et al. 2015). Moreover, therapy resistance is another central matter in patients with AML (Mecklenbrauck and Heuser 2023). Thus, the 5-year overall survival rate of AML patients is about 31.7% based on the National Cancer Institute SEER Cancer Stat Facts: AML (accessed on 18 August 2023). Furthermore, the prognosis of patients with AML progressively worsens with age, until a 5-year survival of 9% in patients aged 65 years and older (Miller et al. 2022). Therefore, dissolving the pathogenesis of AML and further identifying the druggable targets are critically necessary for the clinical development of AML treatment.

Abnormal energy metabolism with weakened mitochondrial aerobic metabolism and overactive glycolysis is widely found in tumor cells during the process of tumorigenesis, which is proposed as the “Warburg effect” (Warburg et al. 1927). In order to meet the needs of rapid proliferation, tumor cells are subjected to metabolic reprogramming, during which cancer cells prefer to utilize glycolysis to metabolize glucose rather than mitochondrial oxidative phosphorylation to generate more

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adenosine triphosphate (ATP) even if oxygen is adequate (Vaupel et al. 2019). Glycolysis is frequently observed in a variety of malignancies, including AML; thus, an anti-tumor role is achieved by suppressing glycolysis to further repress tumor cell proliferation (Geng et al. 2023). Besides, immune escape is another hallmark of tumors, which refers to the process that tumor cells can proliferate and divide rapidly through escaping the surveillance of the immune system's rather than being vanishing (Iorgulescu et al. 2018). Two major mechanisms are involved in tumor immune escape. The tumor microenvironment can change the function of immune cells to inhibit anti-tumor immune responses (Iorgulescu et al. 2018; Chen et al. 2023). Besides, tumor cells can reduce the expression of self-antigens, cause a blemish in the antigen presentation machinery, and decrease the immunogenicity to avert the attack of the immune system (Iorgulescu et al. 2018; Chen et al. 2023). Therefore, targeting immune escape has been demonstrated to be a significant strategy for the treatment of AML (Damiani and Tiribelli 2022).

Ubiquitin-specific peptidase 25 (USP25) is a type of deubiquitinating enzyme (DUB) that modulates biological processes and signal transduction, like cell proliferation, apoptosis, and deoxyribonucleic acid (DNA) repair (Kee and Huang 2016; He et al. 2017; Kim and Baek 2019). The gene with 25 exons on chromosome 21q11.2 encodes USP25 protein that consists of 1087 amino acids with a molecular weight of 150 kDa (Valero et al. 1999). An increased expression level of USP25 is found in the brain and testis tissues of the embryonic mouse (Zhong et al. 2012). Zhu et al. (2021) summarize the role of USP25 in a broad range of diseases, including neurodegenerative diseases, antiviral immunity, metabolic diseases of muscle and adipocytes, and diabetes. Besides, USP25 is abnormally expressed and participates in the progression of diverse cancers, such as hepatocellular carcinoma (Liu et al. 2023), diffuse large B-cell lymphoma (Yang et al. 2023a), glioma (Tang et al. 2022), pancreatic ductal adenocarcinoma (Nelson et al. 2022), and non-small cell lung cancer (Li et al. 2014; Zhu et al. 2021). Moreover, USP25 ensures proliferation of Philadelphia chromosome-positive leukemia cells (Shibata et al. 2020). Nevertheless, the detailed role and mechanism of USP25 in AML are still undiscovered. Furthermore, three ubiquitin-binding domains have been identified at the N terminus of USP25 (Denuc et al. 2009), which make it play the role of deubiquitination in different diseases. For instance, USP25 deubiquitinates transforming growth factor- β activated kinase-1 (TAK1)-TAK1 binding protein 2 (TAB2) after cerebral ischemic stroke to suppress neuroinflammatory responses (Li et al. 2023). USP25 decreases K63-linked polyubiquitination of SMAD family member 4 (SMAD4) in Ang II-induced hypertensive mice to attenuate renal fibrosis (Zhao et al. 2023). USP25 promotes malignant progression by the deubiquitination of murine double minute 2 (MDM2) in diffuse

large B-cell lymphoma (Yang et al. 2023a). Similar findings are also discovered in the pancreatic ductal adenocarcinoma by deubiquitinating hypoxia-inducible factor (HIF)-1 α (Nelson et al. 2022). In the present study, our preliminary experimental results predict that USP25 can deubiquitinate c-Myc based on the UbiBrowser online website. Myc, as a transcription factor, serves a crucial role in cell growth through its ability to trans-activate gene expression. Myc is often activated in AML and participates in the induction of leukemia initiation (Zhou et al. 2021). Thus, according to the above findings, we conjectured that USP25 might mediate the progression of AML through the deubiquitination of c-Myc.

To verify this speculation, AML cell lines and tumor-bearing mice with HL-60 cells were used to explore the function of USP25 in AML. We found the expression of USP25 was increased in AML both *in vitro* and *in vivo*. USP25 enhanced glycolysis and immunity in AML at the molecular level. Mechanically, USP25 promoted proliferation and glycolysis via deubiquitinating c-Myc in AML cells. Collectively, USP25 facilitated proliferation, glycolysis, and immunity through deubiquitinating c-Myc in AML, which indicated that USP25 might be a potential target for AML treatment.

2. Materials and Methods

2.1. Analysis of the USP25 expression *in silico*

The USP25 expression in AML samples and para-carcinoma normal samples was analyzed by the GEPIA (<http://gepia2.cancer-pku.cn>). The overall survival of patients with AML based on the expression of USP25 was determined through the GEPIA database.

2.2. Blood specimens

The peripheral blood specimens from 20 patients with AML were collected, and the patients with AML were confirmed by the French-American-British (FAB) diagnostic criteria. Besides, the peripheral blood samples from 10 healthy volunteers were collected as the control samples. Peripheral blood mononuclear cells (PBMCs) were enriched and purified from AML peripheral blood and control peripheral blood by standard Ficoll-Hypaque density centrifugation methods. All the patients with AML and healthy volunteers were from our hospital, and patients with AML had no other types of cancers. This project was permitted by the Board and Ethics Committee of Guizhou Medical University, and all participants provided the written informed consent (Approval 2024-200).

2.3. Cell culture

Human bone marrow stromal cells HS-5 (Catalog No.: CRL-11882), and AML cell lines KG-1 (catalog number:

CCL-246) and HL-60 (Catalog No.: CCL-240) were obtained from American type culture collection (ATCC; Manassas, VA, USA). HS-5 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Catalog No.: 30-2002, ATCC) with 10% fetal bovine serum (FBS; Catalog No.: 30-2020, ATCC), and KG-1 and HL-60 cells were cultured in Iscove's Modified Dulbecco's Medium (Catalog No. 30-2005) with 20% FBS (Catalog No.: 30-2020, ATCC) at 37°C with 5% carbon dioxide (CO₂). AML cells lines OCI-AML-5 cells (Catalog No.: CBP60528) and MOLM-14 cells (Catalog No.: CBP60678) were bought from Cobioer (Nanjing, China). OCI-AML-5 cells were grown in 80% minimum essential medium α (MEM α ; Catalog No.: 32561037, Gibco, Rockville, MD, USA) with 20% FBS and 10 ng/mL granulocyte macrophage colony-stimulating factor (Catalog No.: G5035, Sigma-Aldrich, St. Louis, MO, USA) with 5% CO₂ at 37°C. MOLM-14 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640 Medium) (Catalog No.: 30-2001, ATCC) with 20% FBS at 37°C with 5% CO₂.

2.4. Cell transfection

The small interfering ribonucleic acid (siRNA) against USP25 (si-USP25) and MYC proto-oncogene (c-Myc) (si-c-Myc), as well as the negative control (si-NC) were acquired from GenePharma (Shanghai, China) and infected into KG-1 and HL-60 cells to diminish the expression of USP25 and c-Myc. The sequences of USP25 were embedded into pcDNA vector plasmids, and then infected into KG-1 and HL-60 cells to elevate the level of USP25. All transfections were conducted by Lipofectamine 3000 (Catalog No.: L3000001, Invitrogen, Carlsbad, CA, USA). After 48 h of the transfection, cells were collected for the subsequent experiments.

2.5. Cell counting kit-8 experiment

Transfected cells with a density of 5×10^3 were sown into 96-well plates. 10 μ L cell counting kit-8 (CCK-8) reagents (Catalog No.: CA1210, Solarbio, Beijing, China) were mixed and incubated at 37°C for 2 h. The absorbance at 450 nm was read under a microplate reader from Thermo Fisher Scientific (Waltham, MA, USA).

2.6. Flow cytometry

Transfected cells were collected and rinsed with cold phosphate buffer saline (PBS; Catalog No.: P1020, Solarbio). Then, cells were mixed into 1 mL of binding buffer, and dyed with propidium iodide (PI) and fluorescein isothiocyanate (FITC)-Annexin V (both from Thermo Fisher Scientific, Waltham, MA, USA) in the dark. After 15 min, cells were determined on a FACScan flow cytometry (BD Biosciences, NJ, USA), and the apoptotic cells were determined by BD CellQuest Pro software (version 5.1, BD Biosciences, NJ, USA).

2.7. Detection of glucose consumption and lactate production

The relative level of glucose consumption and lactate production in transfected cells was detected by the Glucose Assay Kit (Catalog No.: ab65333, Abcam, Cambridge, UK) and Lactate Assay Kit (Catalog No.: MAK064, Sigma-Aldrich, St. Louis, MO, USA) based on the manufacturer's instructions.

2.8. Examination of extracellular acidification rate and oxygen consumption rate

Transfected cells were inoculated in Seahorse XF 96 cell culture microplates with 1×10^4 cells in Iscove's Modified Dulbecco's Medium with 20% FBS. The extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were examined with the Seahorse XF Glycolysis Stress Test Kit (Catalog No.: 103020-100, Seahorse Bioscience, Chicopee, MA, USA) on an XF96 Extracellular Flux analyzer (Seahorse Bioscience, Chicopee, MA, USA) with the Seahorse XF-96 Wave software. For the detection of ECAR, glucose (10 mM), oligomycin (1.0 μ M), and 2-deoxy-D-glucose (2-DG, 50 mM) were successively filled into each well at 20, 40, and 60 min, respectively. For the detection of OCR, oligomycin (2.0 μ M), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (2.0 μ M), and Rotenone antimycin A (Rote + AA, 0.5 μ M) were consecutively filled into each well at 20, 40, and 60 min, respectively. The ECAR and OCR values were measured every 6 min.

2.9. Co-immunoprecipitation experiment

KG-1 and HL-60 cells were administered with the lysis buffer (Catalog No.: R0100, Beijing, Solarbio, China) with protease inhibitors (Catalog No.: A8260, Solarbio, Beijing, China) on ice for 40 min to collect the supernatant. The antibodies against USP25 (Catalog No.: sc-398414, Santa Cruz, Shanghai, China), c-Myc (Catalog No.: ab32072, Abcam, Cambridge, UK), and immunoglobulin G (IgG) (Catalog No.: ab172730, Abcam, Cambridge, UK) were mixed and incubated at 4°C overnight. The mixtures were then incubated with protein A/G-agarose beads (Catalog No.: 78609, Thermo Fisher Scientific, Waltham, MA, USA) and swayed at 4°C. The pelleted cells were harvested and washed with lysis buffer. The precipitates were boiled with loading buffer (Catalog No.: P1040, Solarbio, Beijing, China) for 5 min and determined by Western blot.

2.10. Ubiquitination assay

Transfected cells were lysed with the cell-permeable proteasome inhibitor MG132 (10 μ M; Catalog No.: C2211, Sigma-Aldrich, St. Louis, MO, USA) for 4 h, and then treated with lysis

buffer. The lysates were immunoprecipitated with antibodies against c-Myc (Catalog No.: ab32072, Abcam, Cambridge, UK) or anti-ubiquitin antibody (Catalog No.: 10201-2-AP, Proteintech, Rosemount, MN, USA) at 4°C overnight. Then, the mixtures were hatched with Protein A-Sepharose beads (Catalog No.: ab193256, Abcam, Cambridge, UK) and jiggled for 2 h at 4°C. The beads were rinsed with lysis buffer, and the immunoprecipitated proteins were detected with anti-c-Myc or anti-ubiquitin antibody.

2.11. Cycloheximide assay

To block *de novo* protein synthesis, HL-60 and KG-1 cells transfected with si-USP25 or si-NC were treated with cycloheximide (100 µg/mL; Catalog No.: 239763-M, Sigma-Aldrich, St. Louis, MO, USA) for 0, 1, and 2 h, respectively. Cells were then lysed with RIPA buffer (Catalog No.: R0010, Solarbio, Beijing, China) with protease inhibitors, and used for Western blot detection.

2.12. Real-time quantitative polymerase chain reaction

In keeping with the previous descriptions (Sun et al. 2021; Manríquez-Olmos et al. 2022), the relative mRNA level of USP25 and c-Myc in PBMCs was assessed by Real-time quantitative polymerase chain reaction (RT-qPCR). Total RNA from AML PBMCs and control PBMCs was extracted with TRIzol reagent (Catalog No.: 15596018, Invitrogen, Carlsbad, CA, USA), and reverse transcribed into cDNA by Bio-Rad Scrip™ cDNA Synthesis Kit (Catalog No.: 1708890, Bio-Rad, Hercules, CA, USA) according to the working instructions. RT-qPCR assays were conducted on the Bio-Rad CFX Manager software (Bio-Rad, Hercules, CA, USA) through a 20 µL system (1 µL of the cDNA templates, 1 µL of the 10 µM primers, 10 µL of 2 × SYBR Green polymerase chain reaction (PCR) Mastermix (Catalog No.: SR1110, Solarbio, Beijing, China), and 8 µL of diethyl pyrocarbonate (DEPC) ddH₂O). The primer sequences were 5'-GATTCCTGGCTGTGGGAGTACT-3' (USP25 forward), 5'-TTCTAGGCACTCATGCAGATCTTT-3' (USP25 reverse), 5'-AAAGGCCCCCAAGGTAGTTA-3' (c-Myc forward), 5'-TTTCCGCAACAAGTCCTCTT-3' (c-Myc reverse), 5'-GAAGATCAAGATCATTGCTCC-3' (β-actin forward), and 5'-TACTCCTGCTTGCTGATCCA-3' (β-actin reverse).

2.13. In vivo experiment

To explore the role of USP25 in the growth of AML, 4-week-old bagg albino/c (BALB/c) nude mice were stochastically allotted into short hairpin ribonucleic acid (RNA) negative control (sh-NC) group and short hairpin RNA against USP25 (sh-USP25) group (*n* = 6). Mice in the sh-USP25 group were subcutaneously injected with a total of 3×10^6 of HL-60 cells (Dong et al.

2021) transfected with sh-USP25 into the right flank of nude mice, whereas mice in the sh-NC group were subcutaneously treated with the same amount of HL-60 cells with sh-NC. Tumor volume was monitored 5 days for 4 consecutive weeks and analyzed by the formula: volume = $0.5 \times \text{length} \times \text{width}^2$. Following 4 weeks, mice were sacrificed by intraperitoneal administration of sodium pentobarbital (100 mg/kg). Tumor samples were ablated, weighed, and stored for the Western blot and immunohistochemistry assays.

To explore the role of USP25 in tumor immunity, C57BL/6J mice (*n* = 6) were administered with 5 Gy myeloablative irradiation for 4 h, and then intravenously received 5×10^6 C1498 cells transfected with sh-USP25 or sh-NC through the tail vein, based on the previous report (Dong et al. 2020). Following 15 days, mice were sacrificed by intraperitoneally administering sodium pentobarbital (100 mg/kg). Tumor samples were ablated and stored for the immunofluorescence assays.

Stable HL-60 cells and C1498 cells were constructed with lentivirus carrying short-hairpin RNA targeting USP25 by GenePharma. BALB/c nude mice and C57BL/6J mice were obtained from Cyagen (Jiangsu, China), and kept in a specified pathogen-free condition with the controlled temperature and a 12-h cycle of light-dark. All animal projects were complied with the Guide for the Care and Use of Laboratory Animals (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011) and were authorized by the Animal Research Ethics Committee of Guizhou Medical University (Approval No. 2400472).

2.14. Immunohistochemistry

Tumor tissues were fixed in 4% paraformaldehyde and dehydrated with gradient ethanol. Tissues were immersed into paraffin (Catalog No.: YA0011, Solarbio, Beijing, China) to section into slices (5 µm). The restoration was executed using sodium citrate buffer (pH 6.0; Catalog No.: P0081, Beyotime, Shanghai, China) for 15 min at 94°C. Subsequently, slices were sealed with 1% bovine serum albumin (BSA; Catalog No.: ST2249, Beyotime, Shanghai, China) for 1 h, and managed with antibodies targeting ki-67 (1:1000, Catalog No.: ab15580, Abcam, Cambridge, UK) at 4°C overnight. The secondary antibody HRP labeled anti-rabbit IgG antibody (Catalog No.: ab288151, Abcam, Cambridge, UK) was used to incubate with slices at 37°C for half an hour. The slices were re-stained with hematoxylin (Catalog No.: G1080, Solarbio, Beijing, China) and pictured under a light microscope (Olympus, Tokyo, Japan).

2.15. Immunofluorescence

Immunofluorescence assays were conducted in keeping with the previous study. Mice were perfused with pre-cold 0.1 M PBS (Catalog No.: P1020, Solarbio, Beijing, China)

through the heart and then perfused with pe-cold 4% paraformaldehyde. Tumor tissues were removed to fix into 4% paraformaldehyde overnight, and then embedded in OCT (Catalog No.: 4583, SAKURA, CA, USA). Embedded tissues were sectioned into slices (5 μ m). Slices were blocked with BSA blocking buffer (Catalog No.: SW3015, Solarbio, Beijing, China) and permeabilized with 0.2% Triton X-100 (Catalog No.: T8200, Solarbio, Beijing, China). Subsequently, slices were administered with the antibodies against cluster of differentiation 4 (CD4) (1:50, Catalog No.: ab288724, Abcam, Cambridge, UK) and cluster of differentiation 8 (CD8) (1:100, Catalog No.: MA5-18153, Invitrogen, Carlsbad, CA, USA) at 4°C overnight. The slices were rinsed with PBS thrice and hatched with Goat Anti-Rabbit IgG H&L (HRP) (Alexa Fluor® 647) (1:500, Catalog No.: ab150079, Abcam, Cambridge, UK), or HRP (Alexa Fluor® 488) (1:1000, Catalog No.: ab150157, Abcam, Cambridge, UK) at room temperature for 1 h. Finally, slices were dyed with Mounting Medium, antifading (with 4',6-diamidino-2-phenylindole (DAPI)) (Catalog No.: S2110, Solarbio, Beijing, China) and imaged by fluorescence microscopy (Catalog No.: IX71, Olympus, Tokyo, Japan).

2.16. Western blot

Western blot experiments were executed as in the previous studies. Tumor tissues and cells were lysed with radio immunoprecipitation assay (RIPA) lysis buffer (Catalog No.: R0010, Solarbio, Beijing, China) to extract the total proteins. The bicinchoninic acid (BCA) kit (Catalog No.: PC0020, Solarbio, Beijing, China) was employed to quantify the concentration of the total proteins. Proteins (20 μ g) were electrophoresed with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and shifted onto polyvinylidene fluoride (PVDF) membranes (Catalog No.: IPVH00010, EMD Millipore, Billerica, MA, USA) for the routine operations of Western blot experiments. The membranes were developed with enhanced chemiluminescence (ECL) Western Blotting Detection Kit (Goat IgG) (Catalog No.: SW2030, Solarbio, Beijing, China), and the band intensity was determined by ImageJ software (National Institutes of Health, USA). The primary antibodies included anti-USP25 (1:10000, Catalog No.: ab187156), anti-glucose transporter type 1 (GLUT1; 1:2500, Catalog No.: ab14683), anti-hexokinase-2 (HK2) (1:20000, Catalog No.: ab227198), anti-c-Myc (1:1000, Catalog No.: ab32072), anti programmed cell death ligand 1 (PD-L1) (1:1000, Catalog No.: ab233482), anti- β -actin (1:1000, Catalog No.: ab8227). The secondary antibodies were HRP (catalog number: ab6721, 1:10000). All the primary and secondary antibodies were from Abcam.

2.17. Statistical analysis

Data were expressed as mean $\pm$ standard deviation (SD). The statistical difference was tested using SPSS 26.0

software (IBM, Armonk, New York, USA) with the Student's *t*-test for data between two groups, and the one-way analysis of variance (ANOVA) for data among more than two groups, followed by a *post hoc* Bonferroni test. $P < 0.05$ meant a significant difference.

3. Results

3.1. High expression of USP25 in AML

In silico results showed an enhancement in the expression of USP25 in the tumor tissues from patients with AML relative to that in the normal tissues ($P < 0.05$) (Figure 1a). Moreover, high expression of USP25 indicated a poor overall survival of patients with AML ($P = 0.048$) (Figure 1b). Besides, the transcriptional and translational expressions of USP25 were enhanced in PBMCs from AML peripheral blood as compared with those from control peripheral blood ($P < 0.001$) (Figures 1c,d). Consistently, the protein level of USP25 was prominently elevated in four AML cell lines (OCI-AML-5, MOLM-14, KG-1, and HL-60) as compared with that in HS-5 cells ($P < 0.001$) (Figure 1e). Furthermore, the protein level of USP25 in KG-1 and HL-60 cells was higher than that in OCI-AML-5 and MOLM-14 cells; thus KG-1 and HL-60 cells were selected for the subsequent studies. Thus, these results suggested that high expression of USP25 in AML indicated a poor overall survival of patients with AML.

3.2. USP25 enhanced proliferation and repressed apoptosis in AML cells

High expression of USP25 indicated that USP25 might play a role in the development of AML. Thus, to investigate the role of USP25 in the AML progression, the expression of USP25 was downregulated and upregulated in HL-60 and KG-1 cells by transfecting with si-USP25 and USP25 overexpressing plasmids (USP25). The expression of USP25 was reduced in both cells transfected with si-USP25 ($P < 0.01$), but increased in both cells transfected with USP25 ($P < 0.001$) relative to that in both cells with the corresponding NC (Figure 2a). Transfection with si-USP25 prominently decreased the cell viability ($P < 0.05$), while transfection with USP25 resulted in the inverse outcomes ($P < 0.05$) in both cells (Figure 2b). Conversely, the apoptosis rate in both cells transfected with si-USP25 was elevated ($P < 0.001$), whereas that was prominently declined in both cells transfected with USP25 ($P < 0.01$) (Figure 2c). Therefore, USP25 promoted proliferation but suppressed apoptosis in AML cells.

3.3. USP25 accelerated glycolysis in AML cells

In addition, the role of USP25 in glycolysis was addressed in transfected KG-1 and HL-60 cells. The levels of glucose

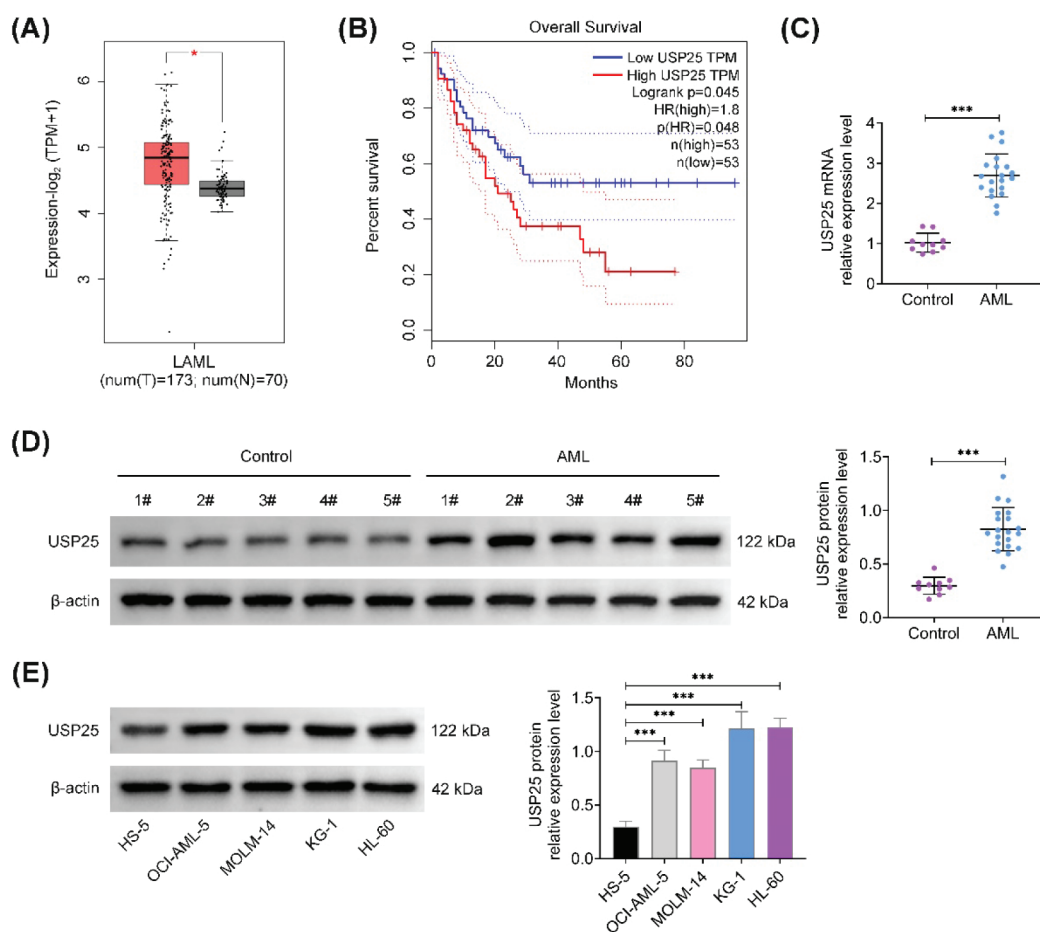


Fig 1. High expression of USP25 in AML indicated a poor overall survival of patients with AML. (A) Upregulation of USP25 in patients with AML based on the data from the The Cancer Genome Atlas (TCGA) database. (B) The overall survival of patients with AML was analyzed based on the expression of USP25. (C) The relative mRNA expression of USP25 in PBMCs from AML or control peripheral blood was detected by RT-qPCR. Data were expressed after being normalized with β -actin. (D) The relative protein expression of USP25 in PBMCs from AML or control peripheral blood was examined by Western blot. Data were expressed after being normalized with β -actin. (E) The relative protein expression of USP25 in human bone marrow stromal cells HS-5 and four AML cell lines was determined by Western blot. Data were expressed after being normalized with β -actin. * $P < 0.05$ and *** $P < 0.001$. AML, acute myeloid leukemia; PBMCs, peripheral blood mononuclear cells; RT-qPCR, real-time quantitative polymerase chain reaction; USP25, ubiquitin-specific peptidase 25.

consumption and lactate production were prominently declined in both cells with si-USP25 ($P < 0.01$), while these were significantly increased in both cells infected with USP25 ($P < 0.05$) (Figure 3a). Besides, transfection with si-USP25 induced a prominent decrease in ECAR and a remarkable increase in OCR in both cells ($P < 0.05$), while transfection with USP25 resulted in an inverse outcome in the ECAR and OCR in both cells ($P < 0.05$) (Figure 3b). Moreover, transfection with si-USP25 diminished the protein level of GLUT1 and HK2 ($P < 0.01$), while transfection with USP25 significantly elevated the protein level of GLUT1 and HK2 ($P < 0.05$) in both cells (Figure 3c). Moreover, to confirm the causal connection between enhanced glycolysis by USP25 and the promoted proliferation by USP25, the investigation of whether glycolysis inhibition by 2-DG rescues the proliferation phenotype induced by USP25

overexpression was explored. The results showed that the cell viability of KG-1 and HL-60 cells was increased following the transfection of USP25, which was counteracted by the treatment of 2-DG ($P < 0.05$) (Figure 3d). Together, USP25 enhanced glycolysis in AML cells.

3.4. USP25 deubiquitinated c-Myc and promoted the expression of PD-L1 in AML cells

Mechanically, the expression of c-Myc, a potential deubiquitination substrate of USP25, was first examined in transfected HL-60 and KG-1 cells. The protein level of c-Myc was markedly declined with the transfection of si-USP25 ($P < 0.01$), and elevated with the transfection of USP25 ($P < 0.01$) in both cells (Figure 4a). Co-immunoprecipitation (Co-IP) results revealed a direct interaction between USP25

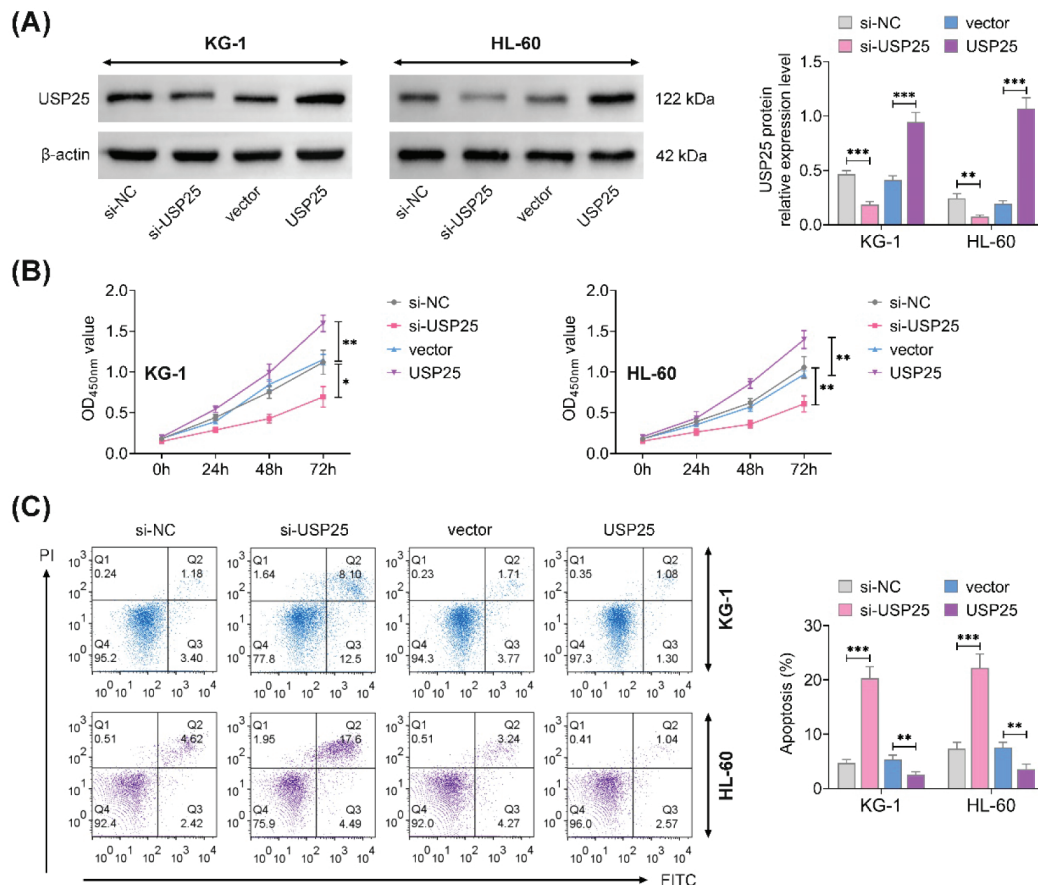


Fig 2. USP25 promoted proliferation, but suppressed apoptosis in AML cells. si-USP25 and USP25 were transfected into KG-1 and HL-60 cells to downregulate and upregulate the expression of USP25 by Lipofectamine 3000. After 48 h of the transfection, cells were collected for the experiments. **(A)** The relative protein expression of USP25 was examined by Western blot. Data were expressed after being normalized with β -actin. **(B)** The cell viability was detected by CCK-8 assays. **(C)** The apoptosis rate was determined by flow cytometry. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. AML, acute myeloid leukemia; CCK-8, cell counting kit-8; USP25, ubiquitin-specific peptidase 25.

and c-Myc (Figure 4b). Moreover, si-USP25-mediated knockdown obviously enhanced polyubiquitination of c-Myc, and USP25-mediated overexpression distinctly decreased polyubiquitination of c-Myc (Figure 4c). Furthermore, the degradation of endogenous c-Myc was expedited in both cells transfected with si-USP25 in a time-dependent way ($P < 0.05$) (Figure 4d), indicating that USP25 promoted the protein stability of c-Myc. More importantly, knockdown of c-Myc decreased the expression of PD-L1, a pivotal protein involved in immune escape that could be modulated by c-Myc, in HL-60 and KG-1 cells with upregulated USP25 ($P < 0.001$) (Figure 4e). The transcriptional and translational expression of PD-L1 was prominently decreased in both cells transfected with si-USP25 and elevated in both cells transfected with USP25 ($P < 0.01$) (Figure 4a). In addition, the relative mRNA expression of c-Myc and PD-L1 was elevated in PBMCs from AML peripheral blood as compared with those from control peripheral blood ($P < 0.001$), and a positive relation was found between USP25 and c-Myc, as

well as between USP25 and PD-L1 (Figure 4f). Altogether, these results suggested that USP25 deubiquitinated c-Myc and promoted the expression of PD-L1 in AML cells.

3.5. USP25 enhanced proliferation and glycolysis via c-Myc in AML cells

To explore whether c-Myc was directly involved in the regulatory role of USP25 in proliferation and glycolysis in AML, HL-60, and KG-1 cells were transfected with USP25 and si-c-Myc. Transfection of USP25 increased the cell viability of both cells, which was significantly counteracted by the transfection of si-c-Myc in a time-dependent fashion ($P < 0.01$) (Figure 5a). The prominent increase in the level of glucose consumption, lactate production, and ECAR, while a significant decrease in the level of OCR in both cells infected with USP25, was markedly reversed with the transfection of si-c-Myc ($P < 0.05$) (Figures 5b,c). Totally, USP25 promoted proliferation and glycolysis via c-Myc in AML cells.

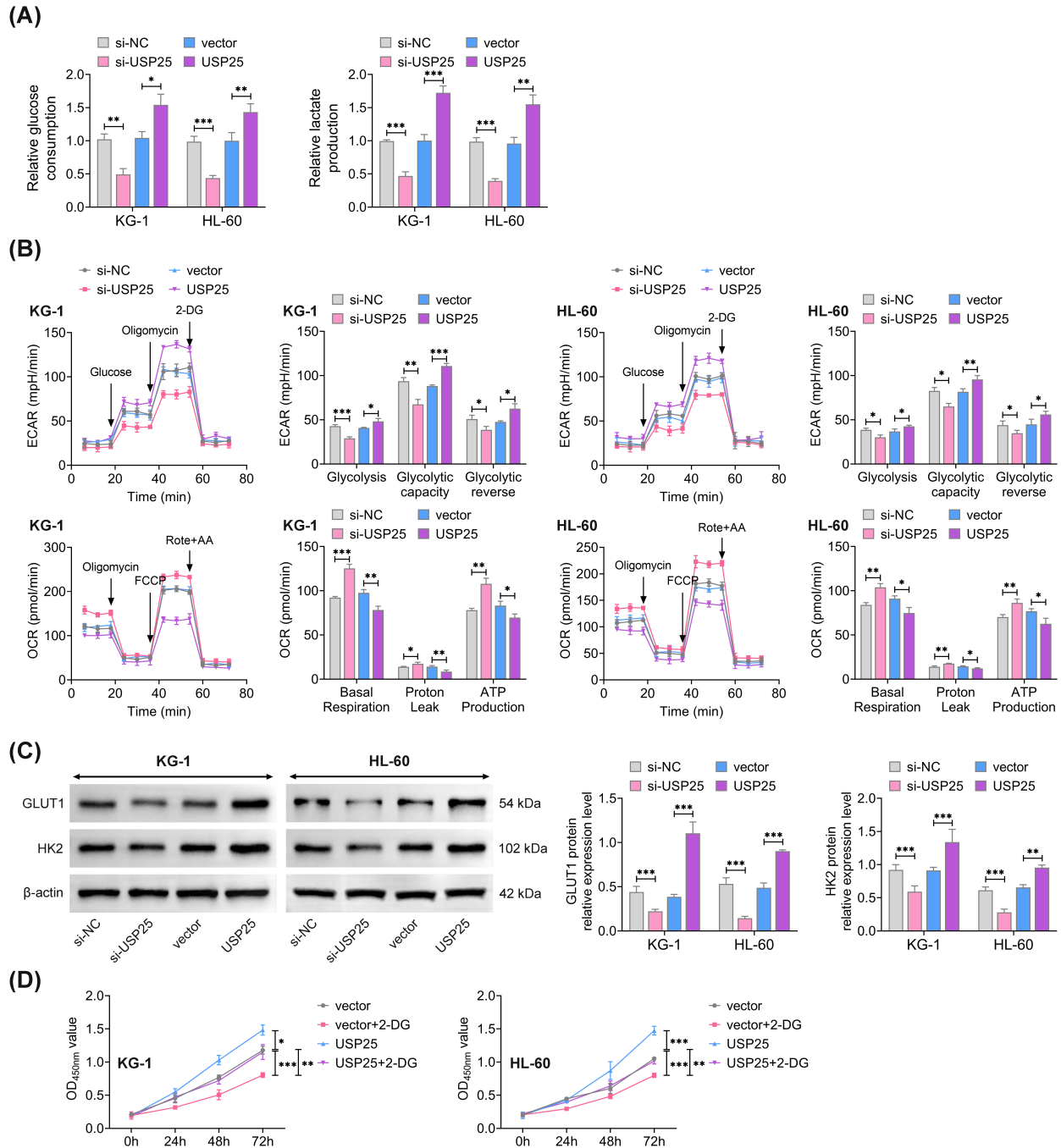


Fig 3. USP25 enhanced glycolysis in AML cells. **(A)** Measurement of the level of glucose consumption and lactate production. **(B)** The ECAR and OCR were detected by an XF96 extracellular flux analyzer. **(C)** The relative protein expression of GLUT1 and HK2 was examined by Western blot. Data were expressed after being normalized with β -actin. **(D)** The cell viability was detected by CCK-8 assays after KG-1 and HL-60 cells were treated with the transfection of USP25 and/or 2-DG. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. 2-DG, 2-deoxy-D-glucose; AML, acute myeloid leukemia; CCK-8, cell counting kit-8; ECAR, extracellular acidification rate; GLUT1, anti-glucose transporter type 1; HK2, hexokinase-2; OCR, oxygen consumption rate; USP25, ubiquitin-specific peptidase 25.

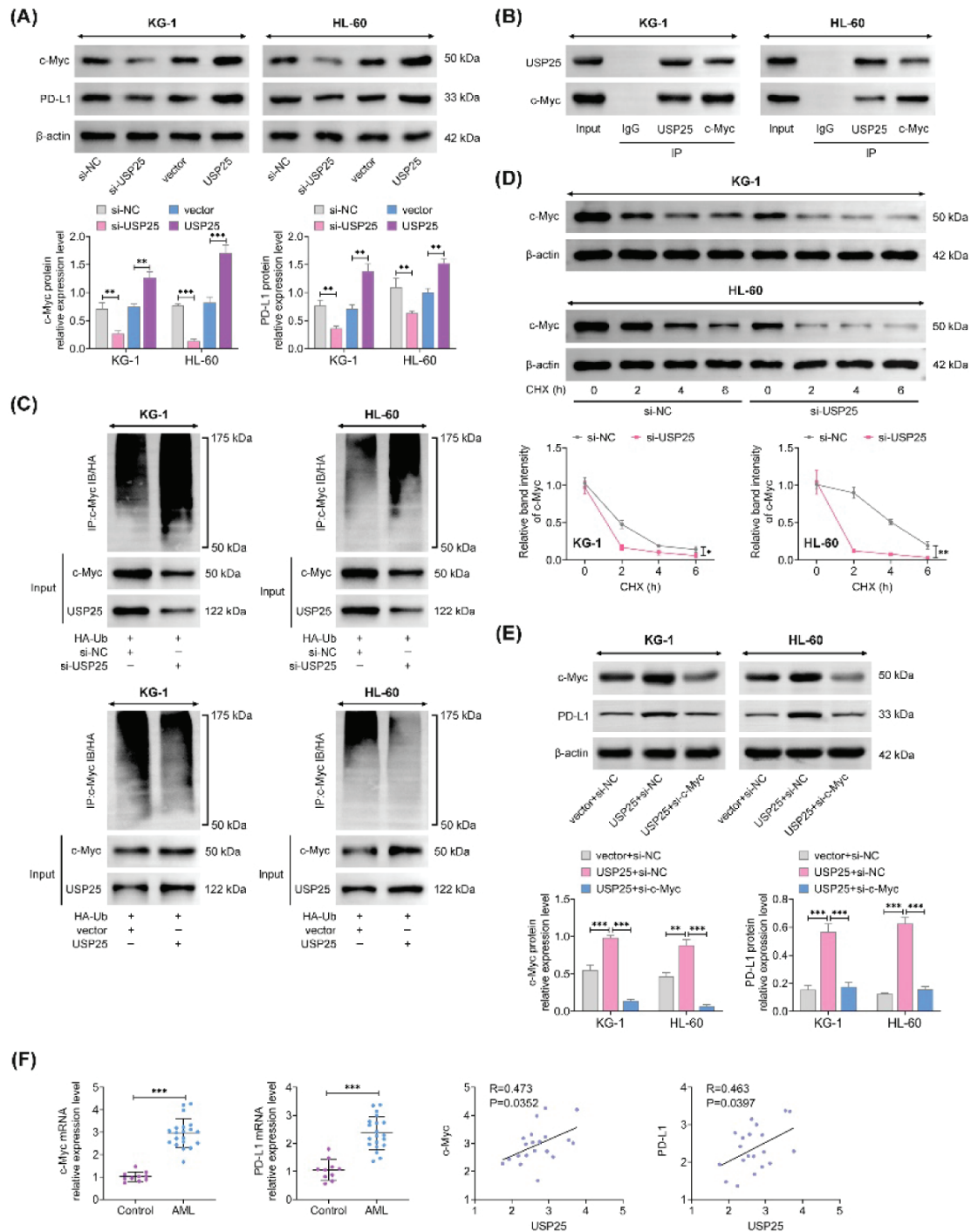


Fig 4. USP25 deubiquitinates c-Myc and increased the expression of PD-L1 in AML cells. **(A)** The relative protein expression of c-Myc and PD-L1 was examined by Western blot after KG-1 and HL-60 cells were transfected with si-USP25, USP25, and their corresponding NC. Data were expressed after being normalized with β -actin. **(B)** The interaction of USP25 and c-Myc was identified by Co-IP assays. **(C)** Determination of the ubiquitination levels of c-Myc. **(D)** The protein stability of c-Myc was examined after KG-1 and HL-60 cells were treated with cycloheximide (CHX). Data were expressed after being normalized with β -actin. **(E)** The expression of PD-L1 in HL-60 and KG-1 cells with upregulated USP25 with or without si-c-Myc was detected by Western blot. Data were expressed after being normalized with β -actin. **(F)** The relative mRNA expression of c-Myc and PD-L1 was detected by RT-qPCR. Data were expressed after being normalized with β -actin. The USP25 expression was positively associated with the c-Myc expression and the PD-L1 expression. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. AML, acute myeloid leukemia; Co-IP, co-immunoprecipitation; RT-qPCR, real-time quantitative polymerase chain reaction; USP25, ubiquitin-specific peptidase 25.

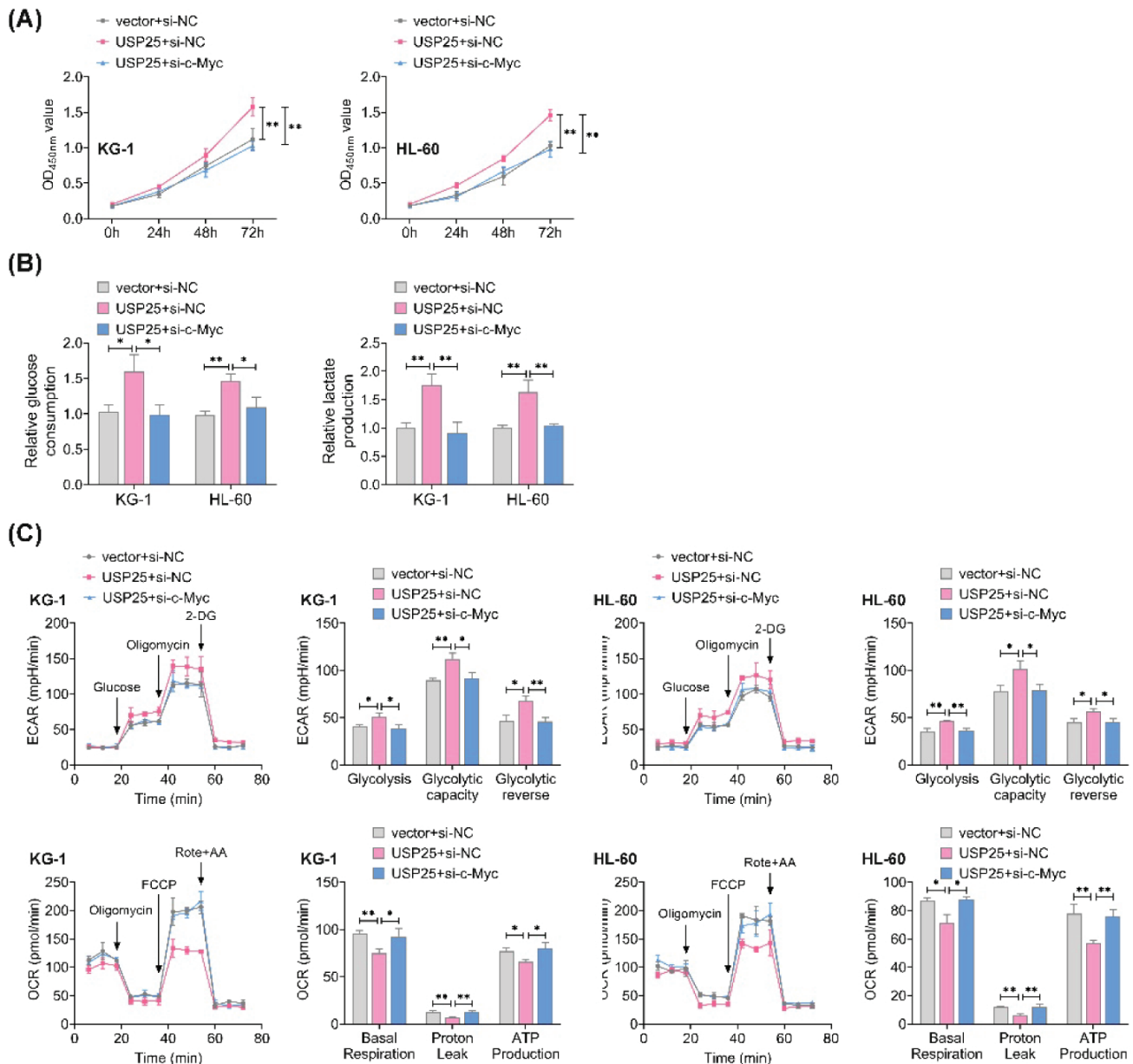


Fig 5. USP25 enhanced proliferation and glycolysis via c-Myc in AML cells. KG-1 and HL-60 cells were transfected with USP25 and/or si-c-Myc, as well as their corresponding NC. After 48 h of transfection, cells were collected for the experiments. (A) The cell viability was detected by CCK-8 assays. (B) Measurement of the level of glucose consumption and lactate production. (C) The ECAR and OCR were detected by an XF96 extracellular flux analyzer. * $P < 0.05$ and ** $P < 0.01$. AML, acute myeloid leukemia; CCK-8, cell counting kit-8; ECAR, extracellular acidification rate; OCR, oxygen consumption rate; USP25, ubiquitin-specific peptidase 25.

3.6. Suppression of USP25 hindered growth and enhanced anti-tumor immunity in tumor-bearing mice

The role of USP25 in the progression of AML was also resolved in BALB/c nude mice inoculated with HL-60 cells with sh-USP25 or sh-NC. The tumor volume and weight in BALB/c nude mice significantly declined with the

sh-USP25-mediated knockdown compared with those in the sh-NC group ($P < 0.001$) (Figure 6a). Knockdown of USP25 ($P < 0.001$) (Figure 6b) in BALB/c nude mice prominently reduced the relative protein expression of c-Myc and PD-L1 in tumor tissues ($P < 0.01$) (Figure 6c). Besides, an obvious decrease in the expression of ki-67 was also found in BALB/c nude mice injected with HL-60 cells transfected with sh-USP25 (Figure 6d). Moreover, to explore the role of USP25

in tumor immunity, C57BL/6J mice intravenously received 5×10^6 C1498 cells transfected with sh-USP25 or sh-NC through the tail vein. The results showed that downregulation of USP25 distinctly enhanced the expression of CD4 and CD8 in tumor tissues ($P < 0.001$) (Figure 6e). Collectively, knockdown of USP25 suppressed growth and enhanced anti-tumor immunity *in vivo*.

4. Discussion

In the current study, the expression of USP25 was elevated in AML, which indicated a poor overall survival in AML patients. Gain- and loss-of-function results exhibited that USP25 enhanced proliferation and glycolysis, but reduced apoptosis in AML cells. Mechanically, USP25 directly bound and deubiquitinated c-Myc. An upregulation of c-Myc was found in AML, and knockdown of c-Myc diminished USP25-induced cell viability, glucose consumption, and lactate production level. Knockdown of USP25 in tumor-bearing mice with

HL-60 cells inhibited tumor growth. Moreover, the level of PD-L1 was elevated in patients with AML, and knockdown of USP25 decreased the expression of PD-L1 both *in vitro* and *in vivo*. Silencing of USP25 in tumor-bearing mice with HL-60 cells increased the level of CD4 and CD8. Taken together, USP25 facilitated proliferation and glycolysis through deubiquitinating c-Myc in AML.

Upregulated expression of USP25 is shown in a variety of cancers, including diffuse large B-cell lymphoma (Yang et al. 2023a), hepatocellular carcinoma (Liu et al. 2023), glioma (Tang et al. 2022), pancreatic ductal adenocarcinoma (Nelson et al. 2022), chronic myelogenous leukemia (Shibata et al. 2020), non-small cell lung cancer (Li et al. 2014), breast cancer (Deng et al. 2007), and stomach adenocarcinoma (Fang and Lu 2020). Similar to these studies, an enhancement in the USP25 expression was observed in the AML *in silico*, *in vitro*, and *in vivo*. Moreover, a high level of USP25 indicated a poor overall survival in patients with AML. Consistently, a high level of USP25 is strongly

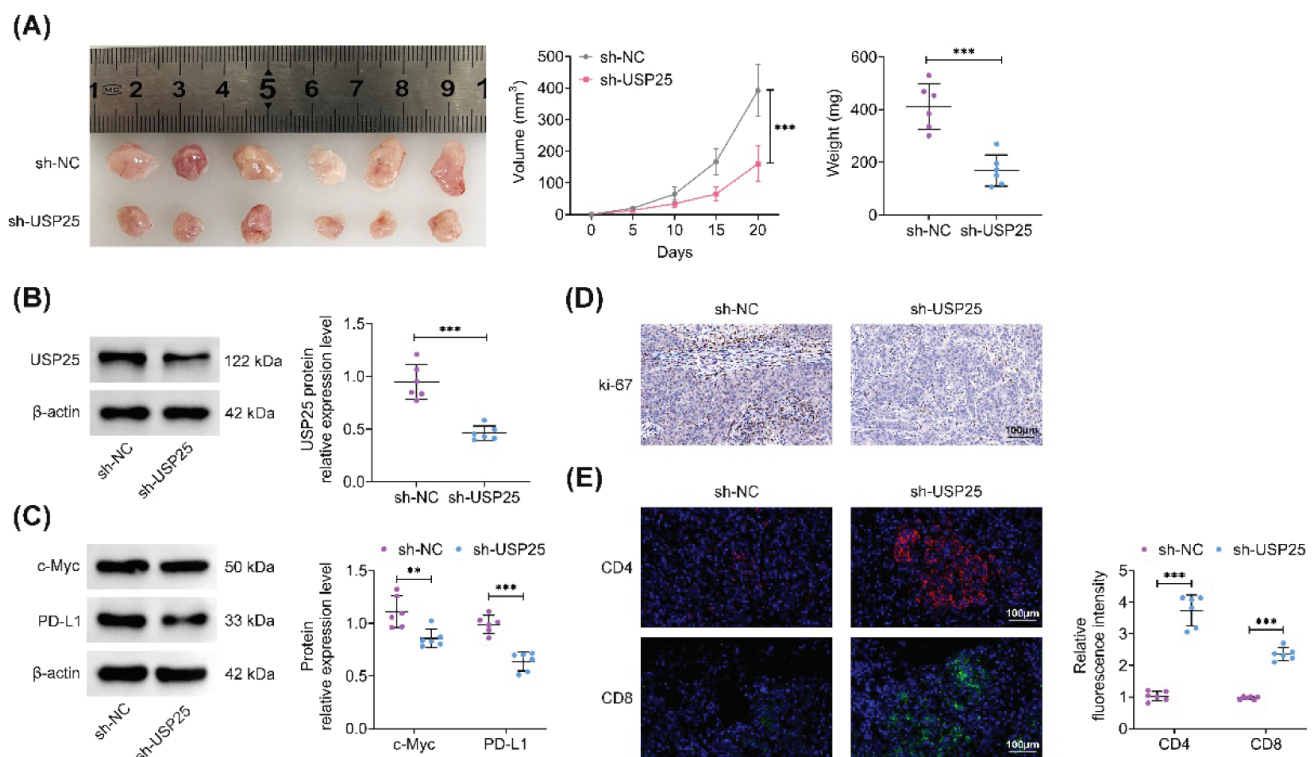


Fig 6. Knockdown of USP25 inhibited growth and facilitated anti-tumor immunity *in vivo*. BALB/c nude mice were subcutaneously inoculated with a total of 3×10^6 of HL-60 cells transfected with sh-USP25 or sh-NC into the right flank of nude mice. Besides, C57BL/6J mice intravenously received 5×10^6 C1498 cells transfected with sh-USP25 or sh-NC through the tail vein. (A) Representative images of neoplasms from BALB/c nude mice (Left), monitoring of tumor volume every 5 days for successive 4 weeks, and calculated according to the following formula: volume = $0.5 \times \text{length} \times \text{width}^2$ (Middle), and measurement of tumor weight (Right). (B) The expression levels of USP25 in tumor tissues from BALB/c nude mice were detected by Western blot. Data were expressed after being normalized with β -actin. (C) The expression levels of c-Myc and PD-L1 in tumor tissues from BALB/c nude mice were examined by Western blot. Data were expressed after being normalized with β -actin. (D) The level of ki-67 in tumor tissues from BALB/c nude mice was determined by immunohistochemistry. Scale bar = 100 μm . (E) The expression levels of CD4 and CD8 in tumor tissues from C57BL/6J mice were examined by immunofluorescence experiments. Scale bar = 100 μm . ** $P < 0.01$ and *** $P < 0.001$. sh-NC, short hairpin RNA negative control; USP25, ubiquitin-specific peptidase 25.

related to poor prognosis in patients with colon, rectum, and stomach adenocarcinoma (Fang and Lu 2020; Wang et al. 2020), hepatocellular carcinoma (Liu et al. 2023), diffuse large B-cell lymphoma (Yang et al. 2023a), and non-small cell lung cancer (Li et al. 2014). Thus, high expression of USP25 in AML indicated a poor overall survival in AML patients. Upregulation of USP25 in different cancers suggests that USP25 may be an oncogene, and suppression of USP25 may inhibit tumorigenesis. This study found that USP25 promoted proliferation but inhibited apoptosis through both gain and loss-of-function assays. Similarly, knockdown of USP25 represses cell proliferation, mobility, and invasion, and overexpression of USP25 causes the inverse outcomes in hepatocellular carcinoma (Liu et al. 2023). Downregulation of USP25 suppresses growth and migration with the increased apoptosis *in vitro*, and inhibits growth and lung metastasis *in vivo* in diffuse large B-cell lymphoma (Yang et al. 2023a). Moreover, knockdown of USP25 also inhibited the growth of AML *in vivo*, as evidenced by the reduced tumor volume and weight, and the level of ki-67. Altogether, high expression of USP25 in AML indicated an unfavorable overall survival in AML patients, and enhanced proliferation with reduced apoptosis in AML. Much energy is demanded for the rapid proliferation and metastasis of tumor cells, which makes energy metabolism greatly important in tumor progression (Fogarty and Hardie 2010; Hardie 2011). Based on the Warburg effect, tumor cells decompose glucose by glycolysis into lactate to achieve the energy supply (Bensinger and Christofk 2012); thus, glycolysis is widely recognized as an energy source for tumor cells (Schwartz et al. 2017). Metabolomics studies discover that enhanced glucose consumption and lactate production, accompanied by the upregulation of several glycolytic enzymes, such as HK2 and GLUT1, are crucial for the occurrence and development of AML (Kreitz et al. 2019), and suppressing glycolysis effectively impedes the progression of AML (Lapa et al. 2020; Dembitz and Gallipoli 2021). Here, overexpression of USP25 elevated the level of glucose consumption, lactate production, and ECAR, and the expression of GLUT1 and HK2, with a decreased OCR level, while knockdown of USP25 resulted in the inverse results in AML cells, indicating that USP25 promoted glycolysis in AML. USP25 has been identified as a potential therapeutic target by enhancing glycolysis in pancreatic cancer (Nelson et al. 2022). In a recent study, USP25 is predicted to modulate aerobic glycolysis and lactate production by M2 isoform of pyruvate kinase, muscle (PKM2) during M1-like polarization, and the USP25-PKM2-aerobic glycolysis axis worsens ischemia reperfusion-induced acute kidney injury (Yang et al. 2023b). An interdependence between tumor metabolism and tumor immune escape has been revealed in plenty researches (Chang et al. 2015; Cascone et al. 2018). Increased glycolysis damages immune elimination of

tumor cells (Cascone et al. 2018), and enhances immune activity in the tumor immune microenvironment in various cancers, including AML (Jiang et al. 2019). Upregulation of immune checkpoint molecules in tumor cells is one of the well-known immune escape mechanisms. Here, the expression of PD-L1 was discovered in patients with AML, and positively associated with the USP25 expression. Knockdown of USP25 reduced the PD-L1 level in AML cells and tumor-bearing mice with HL-60 cells. Furthermore, inhibition of USP25 increased the level of CD4 and CD8 *in vivo*. Thus, USP25 promoted immune activity in AML. Totally, USP25 facilitates glycolysis and immunity in AML. Besides, PD-L1 stimulation has been revealed to enhance the growth and survival of leukemic cells by affecting glucose metabolism in AML (Soltani et al. 2023). Therefore, the role of USP25 in the interplay between glycolysis and immunity should be further addressed in the future.

The significant function of USP25 is the deubiquitination, which has been demonstrated in different diseases. For instance, USP25 deubiquitinates MDM2 in diffuse large B-cell lymphoma (Yang et al. 2023a), Thankyrase 1 (TNKS1) in glioma (Tang et al. 2022), HIF-1 α in pancreatic ductal adenocarcinoma (Nelson et al. 2022), TAB2 in ischemic stroke injury (Li et al. 2023), Kelch-like ECH-associated protein 1 (KEAP1) in liver injury (Cai et al. 2023), SMAD4 in renal fibrosis (Zhao et al. 2023), sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase 2A (SERCA2a) in pathological cardiac hypertrophy (Ye et al. 2023), and Krüppel-like factor 4 (KLF4) in acute pancreatitis (Lv et al. 2022). Analogously, USP25 directly bound and deubiquitinated c-Myc in the present study. Also, USP25 promoted the protein stability of c-Myc in both KG-1 and HL-60 cells. However, the CHX chase in KG-1 cells is weaker than that in HL-60 cells, indicating cell-line variability and needing more research in the future. c-Myc is the most broadly deregulated form of Myc in cancer, which plays a crucial role in restraining apoptosis, and enhancing drug resistance and leukemogenesis in AML (Luo et al. 2005; Pan et al. 2014). Thus, targeting of c-Myc has been revealed as therapeutic strategies for AML treatment (Brondfield et al. 2015; Carabet et al. 2018). Here, the c-Myc level was upregulated in patients with AML, in line with a recent study by Gu et al. (2023). A positive connection was between the c-Myc level and the USP25 level, and knockdown of USP25 reduced the level of c-Myc in AML *in vitro* and *in vivo*. Moreover, suppression of c-Myc decreased USP25-induced cell viability and the glucose consumption and lactate production level in AML cells. Collectively, USP25 facilitated proliferation and glycolysis through deubiquitinating c-Myc in AML.

In summary, our study expounded that USP25 facilitated proliferation and glycolysis through deubiquitinating c-Myc, as well as enhanced immunity in AML. The results provide pre-clinical evidence for the discovery of a potential target for the AML treatment.

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Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Competing Interests

The authors state that there are no conflicts of interest to disclose.

Ethics Approval

All procedures performed in studies involving human participants were in accordance with the standards upheld by the Ethics Committee of Guizhou Medical University and

with those of the 1964 Helsinki Declaration and its later amendments for ethical research involving human subjects (Approval No. 2024-200).

All animal experiments were approved by the Ethics Committee of Guizhou Medical University for the use of animals and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines (Approval No. 2400472).

Statement of Informed Consent

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

Contribution of Authors

Nianxue Wang designed the study, completed the experiment, and supervised the data collection. Can Yang analyzed the data and interpreted the data. Ruya Zhang, Wei Ren, and Biao Shen prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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