

Original Article

EZH2 inhibition overcomes immune evasion in lung adenocarcinoma by restoring CCL5-mediated T-cell recruitment and MHC class I antigen presentation

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Abstract

Immunotherapy represents a pivotal advancement in lung cancer treatment. However, its efficacy remains limited, necessitating a deeper understanding of intrinsic tumor cell mechanisms that modulate the immune micro-environment. Here, we investigate how EZH2 inhibition alters chemokine secretion and antigen presentation in lung adenocarcinoma (LUAD). TCGA and GEO analyses show significant overexpression of *EZH2* in LUAD, which correlates with poorer survival. Immune cell infiltration using MCP-Counter demonstrates that EZH2 expression inversely correlates with CD8⁺ T cell and dendritic cell (DC) infiltration. Spatial analysis of early lung cancer samples reveals an inverse EZH2-CCL5 correlation, suggesting that EZH2 promotes progression via CCL5 suppression. *In vitro* assays included CCK-8 proliferation assays following EZH2 inhibition, qRT-PCR and ELISA to quantify CCL5 expression and secretion, transwell migration assays to assess CD8⁺ T-cell chemotaxis, and co-culture cytotoxicity assays to evaluate MHC class I-dependent T-cell killing. Inhibition of EZH2 markedly reduces tumor cell proliferation, increases CCL5 expression and secretion, enhances CD8⁺ T-cell migration, and promotes MHC class I expression and T-cell-mediated cytotoxicity without altering PD-L1 levels. In conclusion, *EZH2* overexpression promotes immune evasion in LUAD by suppressing CCL5-mediated T-cell recruitment and MHC class I antigen presentation. Targeting EZH2 augments antitumor immunity, supporting its therapeutic potential in combination immunotherapy strategies.

Key words EZH2 inhibitor, non-small cell lung cancer, tumor microenvironment

Introduction

Lung cancer is the most prevalent malignancy worldwide and has the highest mortality rate among all recorded cancer types, with non-small cell lung cancer (NSCLC) constituting the majority of cases. Moreover, the five-year survival rate for lung cancer remains below 20% in most countries [1]. Historically, the primary treatment modalities for NSCLC were limited to surgery, chemotherapy, and radiotherapy. However, with advances in research, targeted therapies and immunotherapies have emerged as groundbreaking treatment options for NSCLC. Nevertheless, a subset of patients still fail to benefit from these therapies, and resistance is

common [2]. In immunotherapy, the phase 3 KEYNOTE-189 study (NCT02578680) 5-year results confirmed that in previously untreated metastatic non squamous NSCLC without EGFR/ALK alterations, pembrolizumab plus pemetrexed-platinum ($n = 410$) vs placebo plus the same chemotherapy ($n = 206$) significantly improved survival: OS HR = 0.60 (95% CI 0.50–0.72), PFS HR = 0.50 (95% CI 0.42–0.60), with 5-year OS rates 19.4% vs 11.3% [3]. Despite the clinical benefits conferred by various therapeutic strategies, including targeted agents and immunotherapy, the toxicities associated with drug combinations and the emergence of drug resistance remain formidable challenges in lung cancer

treatment [4,5]. Therefore, there is an urgent need to develop novel treatment strategies to improve patient outcomes, overcome drug resistance, and ultimately enhance survival rates and quality of life.

EZH2 is a highly activated epigenetic regulator across diverse malignancies. Through PRC2-dependent and -independent methylation, EZH2 suppresses tumor suppressor gene expression and promotes cancer cell proliferation, survival, invasion, and metastasis [6–9]. Elevated EZH2 expression has been widely observed in multiple solid tumors, including breast, lung, colorectal, prostate, and gastric cancers, and is consistently associated with aggressive behavior and poor prognosis. In hematologic malignancies, EZH2 accelerates tumor expansion and dissemination primarily through transcriptional silencing of tumor suppressor genes. Beyond hematologic malignancies, EZH2 also exerts profound oncogenic functions in solid tumors. Extensive studies further demonstrate a pivotal role of EZH2 in metastatic progression, including the promotion of epithelial-mesenchymal transition (EMT), tumor angiogenesis, and distant dissemination.

EZH2 also exerts profound effects on the tumor immune microenvironment. Through H3K27me3-mediated repression, EZH2 downregulates antigen-presentation machinery and other immunostimulatory genes, thereby diminishing tumor immunogenicity [9,10]. Beyond immune regulation, EZH2-driven H3K27me3 also enforces epigenetic lineage control: in pancreatic cancer, EZH2 silences ductal lineage-specifying transcription factors (such as GATA6) and, together with KDM6A loss or hypoxic/Wnt-deficient conditions, shifts cells toward a TP63-driven squamous *trans*-differentiation program that enhances tumor fitness under adverse microenvironmental pressures [11]. At the same time, EZH2 can directly or indirectly increase the expression of immune checkpoint molecules such as PD-L1 and CTLA-4, contributing to impaired T-cell activation [12]. Additional studies show that EZH2, together with DNMT3A and the lncRNA ELFN1-AS1, epigenetically represses MEIS1 transcription to drive oxaliplatin resistance in colorectal cancer and reduces NKG2D ligand expression in glioblastoma stem-like cells to evade NK cell-mediated surveillance [13,14]. EZH2 also contributes to therapy resistance, including PI3K/AKT-driven acquired resistance to gefitinib in NSCLC and cisplatin resistance in ovarian cancer mediated through the c-Myc-miR-137-EZH2 regulatory axis [15,16].

These diverse functions have made EZH2 an attractive therapeutic target, particularly in the context of cancer immunotherapy. Inhibition of EZH2 has been shown to improve responses to immune checkpoint blockade and to enhance the efficacy of multiple adoptive T-cell therapies. In hematologic cancers such as multiple myeloma and acute myeloid leukemia, the EZH2 inhibitor tazemetostat significantly boosts the long-term cytotoxicity and persistence of BCMA- and CD33-directed CAR-T cells [17]. Similar effects have been observed in solid tumors—such as HER2-positive ovarian cancer, prostate cancer, and sarcoma—where EZH2 inhibition strengthens CAR-T and TCR-T-cell-mediated killing. Notably, dual inhibition of EZH1 and EZH2 (e.g., with valemetostat) appears to exert even greater immune-enhancing activity, improving sequential killing, reducing T-cell exhaustion, and producing stronger antitumor effects *in vivo* [18]. Crucially, clinical evidence links EZH2 overexpression to poor prognosis in LUAD [19], yet its impact on key immune evasion mechanisms, such as chemokine-mediated T-cell recruitment and antigen presentation, is unknown.

This study addresses this gap by integrating multi-omics analyses

and functional validation to elucidate how EZH2 inhibition reshapes the LUAD immune microenvironment. However, the immunomodulatory role of EZH2 in the tumor microenvironment of NSCLC remains poorly defined. Here we systematically elucidate the regulatory mechanisms of EZH2 on immune cells and immunomodulatory molecules in LUAD through integrated analysis of bulk RNA-seq and spatial transcriptomic data from public databases combined with experimental validation.

Materials and Methods

TCGA-LUAD bulk RNA-seq and survival modeling

We obtained bulk RNA-seq data and clinical information for LUAD from the TCGA database (<https://portal.gdc.cancer.gov/>). After excluding cases with incomplete survival or clinical data, 513 LUAD patients and 87 normal tissue samples were retained as the training cohort. Differentially expressed genes (DEGs) between LUAD tumor tissues and adjacent normal tissues were identified using the DESeq2 package (v 1.48.1) in R. Raw gene expression counts were imported into a DESeqDataSet object, and a generalized linear model was fitted using a design formula comparing tumor tissues with normal tissues. Data normalization was performed using DESeq2's median-of-ratios method, and differential expression analysis was conducted via the Wald test. DEGs were defined as genes with an absolute $\log_2$ -fold change ($|\log_2FC| > 2$) and an adjusted P value ($P_{adj} < 0.01$), with false discovery rate (FDR) correction applied using the Benjamini-Hochberg procedure.

To visualize the results, a volcano plot was generated using the ggplot2 package, where the x-axis represents $\log_2FC$ and the y-axis represents $-\log_{10}(P_{adj})$. Significantly upregulated ($\log_2FC > 2$, $P_{adj} < 0.01$) and downregulated ($\log_2FC < -2$, $P_{adj} < 0.01$) genes are highlighted in distinct colors. Additionally, key genes of interest (e.g., EZH2) were manually annotated using ggrepel to avoid label overlap. We additionally analyzed LUAD bulk RNA-seq datasets from the GEO database (GSE10072, GSE30219, and GSE31210) to evaluate EZH2 expression differences between LUAD and normal lung tissues. Differential expression analysis was performed using the limma package on $\log_2$ -transformed counts (with a pseudocount of 1). Significantly DEGs were defined as those with $|\log_2FC| > 1$ and $FDR < 0.05$. Heatmaps of DEGs were generated using the R package pheatmap, and Venn diagrams were constructed with the Sanberbox tool [20] (<http://www.sangerbox.com/tool>) (Supplementary Figure S1A). Using the R package maxstat, we determined the optimal cutoff for EZH2 expression (2.462, with group sizes constrained to 25%–75% of samples) and stratified patients into high- and low-expression groups in TCGA-LUAD datasets, observing significant survival differences via Kaplan-Meier analysis and log-rank testing. A Cox-based nomogram integrating survival time, status, and seven prognostic features was constructed (*rms* package), demonstrating strong discrimination. Time-dependent ROC analysis (*pROC* package) was used to evaluate EZH2's predictive power at the 1-, 3-, and 5-year follow-ups. Using the maxstat-derived cutoff, the SEAS risk score (based on stage, EZH2 expression, age, and sex) effectively stratified patients into high- and low-risk groups with significantly different survival outcomes.

Comprehensive tumor microenvironment characterization in LUAD

Using the R package ESTIMATE (version 1.0.13), we calculated stromal, immune, and ESTIMATE scores for each patient based on

gene expression profiles. In the TCGA-LUAD cohort, samples were divided into high- and low-expression groups based on the median expression of EZH2. The R package "GSVA" was used to perform single-sample gene set enrichment analysis (ssGSEA) to evaluate antitumor immune cell infiltration. RNA-seq data were normalized by DESeq2 and $\log_2$ -transformed. Wilcoxon rank-sum tests were conducted to compare infiltration differences between groups (significance threshold: $P < 0.05$), and ggplot2 was used to generate boxplots for visualization.

Samples were divided into high- and low-expression groups based on the median expression level of EZH2. Immune cell infiltration scores for 28 cell types were calculated using the deconvoluted xCell method (xCell) algorithm (implemented via the IOBR package), with $\log_2(x + 1)$ transformation applied to the scores. Wilcoxon rank-sum tests were performed to compare between-group differences, with significance set at $P < 0.05$. Boxplots revealed significant differences in immune cells between groups.

We reassessed the infiltration scores of B cells, CD4⁺ T cells, CD8⁺ T cells, neutrophils, macrophages, and DCs in each patient's tumor based on EZH2 gene expression using the TIMER method implemented in the R software package.

We systematically analyzed immune cell infiltration in the tumor microenvironment using the CIBERSORT algorithm [21]. First, we deconvoluted the proportions of 22 immune cell subsets in each sample based on the LM22 signature matrix and visualized the overall distribution characteristics of immune cell composition across samples using a rainbow plot. Samples were then stratified into high- and low-expression groups according to the median expression level of EZH2, with boxplots used to compare significantly different immune cell subsets between groups (Wilcoxon rank-sum test, $P < 0.05$). Furthermore, based on differential expression analysis results, we selected the top 10 DEGs with the largest $|\log_2FC|$ values, which together with the target gene formed an 11-gene analytical subset. Using the CIBERSORT algorithm, we calculated infiltration scores for 22 immune cell types. Spearman's rank correlation analysis (significance threshold FDR < 0.05) was employed to assess the correlation between the expression levels of these 11 genes and the infiltration levels of various immune cells. The correlation coefficients (ρ) were visualized via the pheatmap package (version 1.0.12), with rows representing the 11 genes and columns representing the 22 immune cells. The color gradient indicates correlation strength (red for positive correlation, blue for negative correlation), and bidirectional clustering was performed using Euclidean distance with complete linkage. Finally, we generated 22 scatter plots to individually illustrate the correlation between infiltration levels of clinically significant immune cell subsets and target gene expression (with correlation coefficient ρ and P value annotated).

Subsequently, employing xCell from the R package IOBR (version 0.99) [22], we comprehensively evaluated immune cell infiltration scores in TCGA LUAD samples, as well as the composite immune score, stroma score, and microenvironment score.

Spatial transcriptomics analysis

For spatial transcriptomics data analysis, we used the publicly available dataset GSE189487 obtained from GEO datasets [23]. The analysis was performed using the Seurat R package (version 4.2). As the original data lacked imaging information, samples were

manually annotated in Seurat based on well-established cell-type markers reported in the literature. Each sample was normalized using the SCTransform method. PCA was employed for dimensionality reduction, followed by the construction of neighbor graphs and clustering based on the top 30 principal components (FindNeighbors and FindClusters, resolution = 0.5), with subsequent visualization through UMAP [24]. All analyses were conducted under the SCT assay.

Human Protein Atlas (HPA) database

Immunohistochemical data for EZH2 expression in normal and LUAD tissues were obtained from The Human Protein Atlas (HPA, <http://www.proteinatlas.org>). Patient clinical information is provided in [Supplementary Table S1](#).

Cell culture

Human normal lung epithelial cell lines and LUAD cell lines (PC9, A549, and H1299) were purchased from ATCC (Manassas, USA). Cells were cultured in DMEM (HyClone, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, USA) and 1% penicillin/streptomycin (Gibco) at 37°C with 5% CO₂. Commercially available human peripheral blood mononuclear cells (PBMCs) were obtained from AllCells (Shanghai, China) and routinely tested for mycoplasma contamination.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from cell pellets using TRIzol reagent (1 mL TRIzol per sample; Invitrogen, Carlsbad, USA), followed by the addition of 200 μ L chloroform and thorough mixing. After 5 min of incubation at room temperature, the mixture was centrifuged at 12,000 g for 15 min at 4°C to separate the aqueous phase. RNA was precipitated with an equal volume of isopropanol by centrifugation at 12,000 g for 10 min at 4°C. The RNA pellet was washed twice with pre-chilled 75% ethanol (7500 g , 10 min at 4°C), air-dried, and dissolved in DEPC-treated water. RNA concentration was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and stored at -80°C.

For reverse transcription, 500 ng of total RNA was used in a 10 μ L reaction system containing 2 μ L of 5 × RT Master Mix, with DEPC-treated water added to the final volume. The synthesized cDNA was used for subsequent experiments. Quantitative real-time PCR (qPCR) was performed using the kit from TaKaRa (Dalian, China) under the following conditions: initial denaturation at 95°C for 5 min, followed by 30 amplification cycles. *GAPDH* served as the internal control, and each sample was analyzed in triplicate. The primer sequences are listed in [Table 1](#).

Western blot (WB) analysis

Cells were washed with PBS, trypsinized (0.25%), and centrifuged to collect pellets. Proteins were extracted using RIPA lysis buffer containing protease inhibitors, followed by centrifugation (12,000 g , at 4°C for 10 min). Protein concentration was determined by BCA assay (1 μ L sample + 19 μ L lysis buffer, incubated with 200 μ L BCA working solution at 37°C for 30 min, OD562 measurement; P0012S, Beyotime, Shanghai, China). Samples were denatured with 5 × loading buffer (boiling, 10 min) and stored at -80°C. SDS-PAGE gels were prepared based on target protein molecular weight. Electrophoresis was initially conducted at 80 V until the dye front reached

Table 1. Sequences of primers used for qRT-PCR

Gene	Forward (5'→3')	Reverse (5'→3')
<i>EZH2</i>	GTACACGGGGATAGA GAATGTGG	GGTGGCGGGCTTTCT TTATCA
<i>CCL5</i>	GCTGCTTTGCCTACCT CTCC	TCGAGTGACAAA CACGACTGC
<i>HLA-A</i>	GATTACATCGCCTTGA ACGAGG	AGAGACAGCGTGGT GAGTCAT
<i>HLA-B</i>	CAGTTCGTGAGGTTC GACAG	CAGCCGTA CATGCTCTGGA
<i>HLA-C</i>	CCATGAGGTATTTGT GGACCG	TCTCGGACTCTCGTC GTCG
<i>B2M</i>	GAGGCTATCCAGCG TACTCCA	CGGCAGGCATACT CATCTTTTT

the stacking/separating gel interface and then increased to 120 V for completion when the dye front approached the gel bottom, followed by wet transfer (90 V, ice bath) to PVDF membranes (pre-activated in methanol for 30 s). Membranes were blocked with 5% skim milk/TBST (2 h; room temperature, RT), incubated with primary antibodies (4°C overnight). The primary antibodies used were: anti-EZH2 (1:1000, Cat. No. ab186006; Abcam, Cambridge, UK), anti-CCL20 (0.2 µg/mL, Cat. No. ab9829; Abcam), anti-CCL5 (1:50, Cat. No. sc-365826; Santa Cruz Biotechnology, Santa Cruz, China), and anti-β-tubulin (1:10,000; Cat. No. T0023; Affinity Biosciences, Cincinnati, USA). After washing with TBST (3 × 5 min), and probed with HRP-conjugated goat anti-rabbit IgG (1:3000, Cat. No. S0001; Affinity Biosciences) or goat anti-mouse IgG (1:3000; Cat. No. S0002, Affinity Biosciences) for 1 h at RT. Protein bands were visualized using ECL substrate (1:1 ratio) and imaged with a chemiluminescence system (Tannon Science & Technology, Shanghai, China).

Lentiviral packaging

293FT cells at 80%–90% confluency were transfected with the target plasmid (EZH2 shRNA, purchased from Shanghai Lingke Biotechnology, Shanghai, China), packaging plasmids (psPAX2), and envelope plasmid (pMD2. G) in Opti-MEM. Viral supernatants were collected at 48 and 72 h post-transfection, filtered (0.45 µm), and stored at –80°C. Target cells (60%–70% confluency) were infected with 1.5 mL viral supernatant containing polybrene (8 µg/mL). Stable clones were selected with puromycin (2 µg/mL) and validated by qPCR and western blot analysis.

ELISA

CCL5 and CCL20 levels in cell supernatants were measured using human ELISA kits, which were purchased from Neobioscience Technology (Cat. No. EHC143.96.2; Shenzhen, China). Samples/standards were diluted 1:100 with specific antibodies. After 12 min of incubation at 37°C and washing, HRP-conjugated streptavidin was added (45 min, RT). TMB substrate was used for color development (30 min, dark), and reactions were terminated before measuring OD₄₅₀–OD₅₇₀.

CD8⁺ T-cell isolation

CD8⁺ T cells were isolated from human peripheral blood mononuclear cells from AllCells using immunomagnetic bead separation. Briefly, PBMCs were resuspended in the separation buffer provided

with the kit (Cat. No. #17953; STEMCELL Technologies, Shanghai, China), and a small aliquot was analyzed by flow cytometry to determine the baseline CD8⁺ T cells population. The cell suspension was then incubated with CD8-specific selection antibodies at room temperature for 3 min to allow antibody-target cell binding. Magnetic beads were subsequently added, mixed gently, and placed in a magnetic stand for 3 min to capture CD8⁺ T cells. Unbound cells were discarded, and the bead-bound CD8⁺ T cells were washed three times with buffer before final resuspension in complete culture medium. The purity of isolated CD8⁺ T cells was verified by flow cytometry, with ≥ 90% purity required for downstream applications.

Flow cytometry

To validate the isolation efficiency, the sorted CD8⁺ T cells were analyzed by flow cytometry for surface marker expression. The fluorophore-conjugated anti-CD8 antibody used was FITC anti-human CD8 (Cat. No. #980908; BioLegend, Beijing, China). Cells were washed with PBS and centrifuged to remove the supernatant. The pellet was resuspended in flow cytometry buffer and stained with fluorophore-conjugated anti-CD8 antibody for 30 min at 4°C in the dark. After incubation, unbound antibodies were removed by two washes with buffer, and the cells were resuspended in 500 µL of buffer for analysis. Flow cytometry was performed, and data were processed using FlowJo software (<https://www.flowjo.com/>) to confirm CD8⁺ T-cell purity.

CD8⁺ T-cell activation

For T-cell receptor (TCR) stimulation, CD8⁺ T cells were activated using antibody-coated plates. Briefly, 24-well plates were coated with anti-CD3 antibody (Cat. No. #05121-25-500; PeproTech, Rocky Hill, USA) at a concentration of 5–10 µg/mL by incubation at 37°C for 2 h or overnight at 4°C. Unbound antibodies were removed by washing three times with PBS. Purified CD8⁺ T cells were then seeded at a density of 1.2 × 10⁶ cells/mL in the coated plates, supplemented with anti-CD28 antibody (Cat. No. #10311-25-500; PeproTech) at 2 µg/mL to provide co-stimulatory signals. Cells were cultured at 37°C with 5% CO₂ for 48–72 h, with periodic monitoring of proliferation. Activated CD8⁺ T cells were harvested for subsequent functional assays or molecular analyses.

Transwell migration assay

To assess CD8⁺ T-cell migration, a Transwell assay was performed. First, cell culture supernatants were collected from the experimental and control groups, centrifuged at 1000 g for 5 min, and filtered. CCL5 was prepared as a stock solution and diluted to the working concentration according to the manufacturer's instructions (Cat. No. 300-06; PeproTech). A 24-well plate was prepared with the following groups (600 µL/well): (1) 1640 medium (negative control), (2) experimental cell supernatant, (3) untreated control supernatant, (4) CCL5 (positive control), and (5) anti-CCL5 antibody group. The Transwell inserts were carefully placed into each well to avoid bubbles. CD8⁺ T cells, isolated from PBMCs and resuspended in 1640 complete medium, were added to the upper chamber. After 8 h of incubation at 37°C, the inserts were removed, and migrated cells in the lower chamber were resuspended and counted using a Countstar automated cell counter (Alit Life Science, Shanghai, China).

Co-culture of CD8⁺ T cells with tumor cells

The cytotoxic activity of activated CD8⁺ T cells was evaluated using LUAD cell lines (PC9 and H1299). Tumor cells were seeded in a 96-well plate at 1×10^4 cells/well and cultured for 24 h. Activated CD8⁺ T cells were then added at a 1:4 effector-to-target (E:T) ratio and co-cultured for 48 h. After incubation, the supernatant was removed, and the wells were washed with PBS to eliminate dead cells and residual CD8⁺ T cells. The remaining tumor cell viability was measured using the CCK-8 assay (detailed below).

CCK-8 assay

Cell proliferation was assessed using the CCK-8 kit (Dojindo, Tokyo, Japan). Cells treated with or without the EZH2 inhibitor GSK126 (Cat. No. S7061; Selleck, Houston, USA) were harvested, washed with PBS, and resuspended in complete medium. A 96-well plate was seeded with 100 μ L of cell suspension per well (triplicates per group), with ddH₂O added to the outer wells to prevent evaporation. At designated time points (24, 48, 72, 96, 120, and 144 h), the medium was replaced with a CCK-8/medium mixture (1:9 ratio, 100 μ L/well). After 2.5 h of incubation, the absorbance at 450 nm was measured using a microplate reader (Epoch, BioTek, USA), and proliferation curves were plotted.

Statistical analysis

Data were analyzed using SPSS 20.0, GraphPad Prism 8.0, and R software (version 4.4.3). Quantitative experimental data are presented as mean $\pm$ standard deviation (SD). For comparisons between two groups, the Student's *t* test was used. For comparisons among multiple groups (e.g., qRT-PCR and co-culture assays), one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed. Bioinformatic analyses were conducted as described in the respective method sections. All statistical tests were two-tailed, and a *P* value < 0.05 was considered statistically significant.

Results

EZH2 is upregulated in LUAD with adverse prognosis and pro-proliferative pathways

TCGA databases indicate that EZH2 exhibits differential expression in various tumor tissues and normal tissues, with significant differences observed in thoracic tumors such as esophageal cancer, lung squamous cell carcinoma, and LUAD (Figure 1A). We identified DEGs in TCGA-LUAD, with EZH2 showing significant differential expression in tumor tissues (Figure 1B). Further analysis revealed 614 consistently upregulated tumor-associated genes shared with three additional GEO datasets, including EZH2 (Supplementary Figure S1A). Among the numerous DEGs, EZH2 was prioritized due to its role as a key epigenetic regulator and a potential therapeutic target in NSCLC. Notably, EZH2 inhibitors have demonstrated promising antitumor activity and the potential to overcome drug resistance [25,26]. Analysis of TCGA data revealed that EZH2 expression was higher in LUAD samples than in normal samples, and the proportion of EZH2 expression was greater in tumor tissues than in adjacent normal tissues (Figure 1C, D). Immunohistochemistry data from public databases showed that LUAD tissues had a higher proportion of EZH2 expression (Figure 1E,F). GO and KEGG analyses of TCGA-LUAD samples demonstrated three core pathogenic mechanisms in LUAD (Supplementary Figure S1B–D). In terms of immune regulation, GO analysis

demonstrated significant activation of the leukocyte-mediated immune response ("leukocyte-mediated immunity"), while KEGG analysis further identified abnormalities in the cytokine-receptor interaction pathway ("cytokine-cytokine receptor interaction"), collectively indicating substantial immune dysregulation in the tumor microenvironment. Regarding metastatic characteristics, GO analysis identified extracellular matrix ("collagen-containing extracellular matrix") remodeling and loss of cell polarity ("apical plasma membrane"), while KEGG analysis revealed activation of cytoskeletal ("cytoskeleton in muscle cells") and ECM-receptor interaction ("ECM-receptor interaction") pathways, jointly driving tumor invasion and metastasis. In metabolic regulation, GO analysis detected ion channel dysfunction ("metal ion transporter"), and KEGG analysis showed alterations in amino acid metabolism ("biosynthesis of amino acids") and arachidonic acid metabolism ("arachidonic acid metabolism") pathways, suggesting significant metabolic reprogramming in tumors. GSEA indicated significant activation of E2F targets and G2/M checkpoint-related pathways in tumor samples, suggesting robust proliferative dysregulation, metabolic reprogramming, and genomic instability in tumor cells (Supplementary Figure S1E–G). This provides a molecular basis for precision therapeutic strategies targeting cell cycle inhibitors and metabolic intervention. EZH2 inhibitors also upregulate super-silencer-controlled genes linked to the cell cycle, apoptosis and DNA damage, exerting *in vivo* anti-cancer effects [27]. GSEA C7 gene set analysis revealed significant enrichment of immunological processes such as dynamic changes in CD8⁺ T-cell effector function and differences in primary/secondary immune responses, highlighting key regulatory nodes in lymphocyte activation, tolerance, and memory formation (Supplementary Figure S1H–J). In the process of transformation from myelodysplastic syndrome (MDS) to acute myeloid leukemia (AML), EZH2 is believed to downregulate CXCL10, thereby affecting CD8⁺ T-cell exhaustion [28]. This may indicate that the role of EZH2 in LUAD may be accompanied by both cell cycle abnormalities and changes in immune cells within the tumor microenvironment.

In the survival analysis, LUAD patients with high EZH2 expression showed a worse prognosis (Figure 1G). A multivariate "SEAS" risk model—incorporating stage, EZH2 expression, age, and sex—demonstrated a concordance index (C-index) of 0.686 (95% CI: 0.635–0.737; *P* value = 6.78×10^{-13}) (Supplementary Figure S2A). Subsequent Kaplan-Meier analysis confirmed statistically significant survival differences between the high- and low-risk groups (*P* value = 4.1×10^{-10}) (Figure 1H). Time-dependent ROC analysis at 365, 1095, and 1825 days validated the model's predictive accuracy, and the results were consistent with the nomogram, confirming the negative correlation of EZH2 with patient survival (Supplementary Figure S2B,C). This model provides a quantifiable tool for clinical prognosis assessment, with potential for performance optimization through incorporation of additional clinical variables. To investigate the functional mechanisms of EZH2 in LUAD, we first stratified samples into high- and low-EZH2 expression groups. Subsequently, GO and KEGG enrichment analyses were carried out. For the GO enrichment analysis (Supplementary Figure S2D), it was revealed that, across biological processes, cellular components, and molecular functions, the DEGs were prominently enriched in cell cycle-associated items such as nuclear division and chromosome segregation. They were also enriched in cell structure-related entries, such as chromosomal region and microtubule, as well as molecular

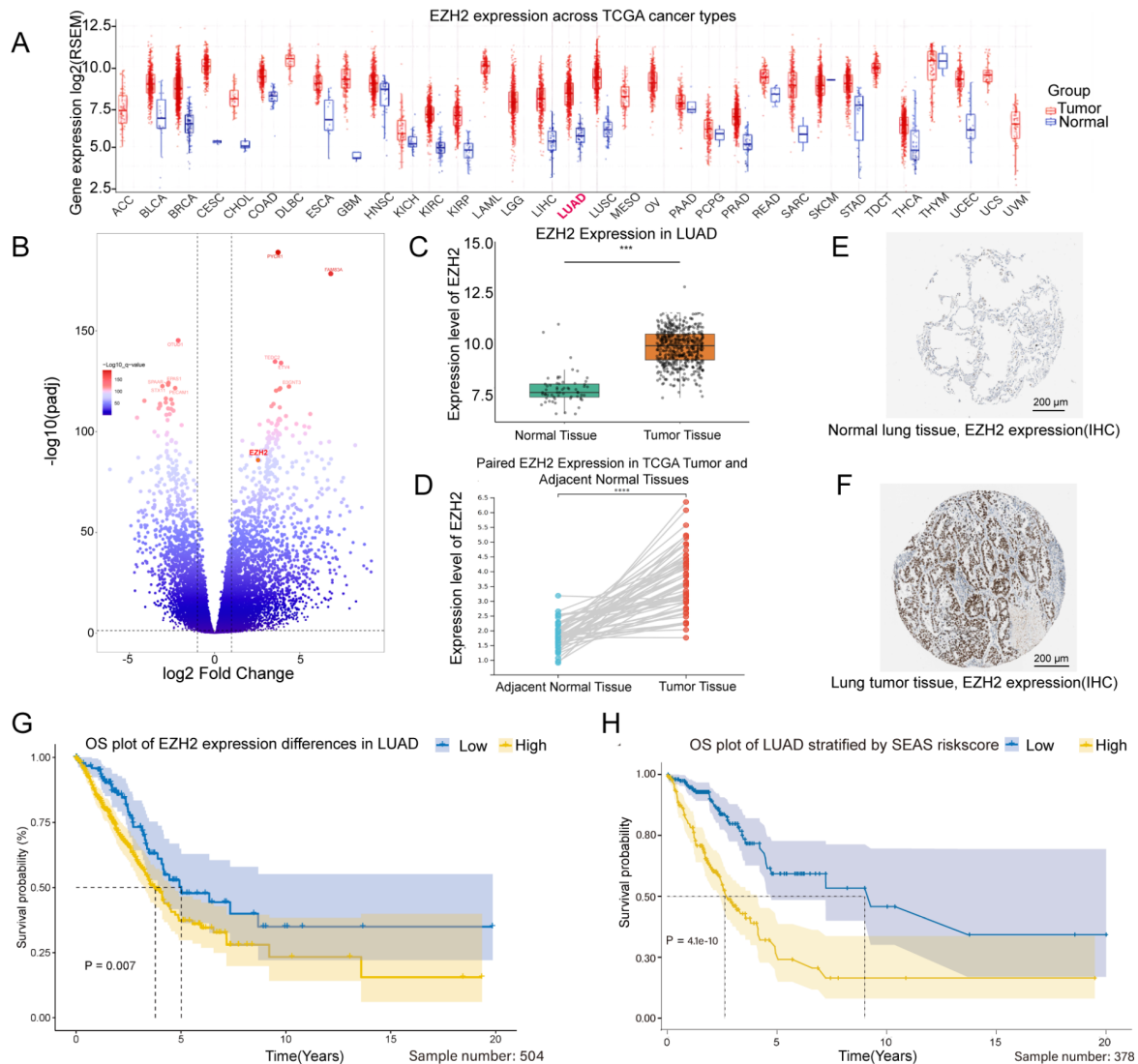


Figure 1. Comprehensive analysis of EZH2 expression in various settings (A) Expression profile of EZH2 in pan-cancer. (B) Volcano plots separately displaying significant DEGs ($|\log_2 FC| > 2$, $P < 0.01$) from TCGA-LUAD. Red points represent upregulated genes, blue points represent downregulated genes, and gray points represent genes with no significant difference. (C) Box plot comparing EZH2 expression in normal and tumor tissues based on TCGA databases. (D) Expression of EZH2 in tumor and adjacent normal tissues of TCGA LUAD patients. (E) EZH2 immunohistochemical (IHC) staining in normal human lung tissue (HPA database). (F) EZH2 immunohistochemical (IHC) staining in LUAD tissue (HPA database), showing increased nuclear staining intensity (strong positivity, 3+) compared to normal tissue. Staining pattern: nuclear localization in malignant cells. (G) Kaplan-Meier survival curves for EZH2 expression in TCGA LUAD. Kaplan-Meier survival analysis with the log-rank test showed a significant overall survival (OS) difference between groups ($P = 0.007$). (H) SEAS riskScore cutoff determined by maxstat, dividing patients into high-risk and low-risk groups.

function-related terms, including microtubule binding and endopeptidase activity. Regarding the KEGG enrichment analysis (Supplementary Figure S2E), the DEGs were mainly enriched in pathways such as neuroactive ligand–receptor interaction and cell cycle. These results suggest that EZH2 is likely to impact the development and progression of LUAD by regulating these functional pathways.

EZH2 is correlated with an immune-suppressive tumor microenvironment in LUAD

Based on the findings from GO, KEGG, and GSEA analyses, we found that EZH2 may influence cancer development through the

action of immune cells. Next, we evaluated the correlation between ESTIMATE scores (ESTIMATE, immune, and stromal scores) and EZH2 expression levels in TCGA-LUAD (Figure 2A). High EZH2 expression was associated with reduced infiltration of immune and stromal cells in LUAD, leading to higher tumor purity. Combined with the XCELL immune scores, the results indicated that EZH2 plays a negative regulatory role in immune modulation in LUAD (Figure 2B). Xcell analysis indicated that EZH2 expression was inversely associated with the abundance of several immune-activating cell types, such as CD4⁺ T cells and dendritic cells. ssGSEA and TIMER analysis revealed that the low-expression group had significantly increased infiltration scores of antitumor immune

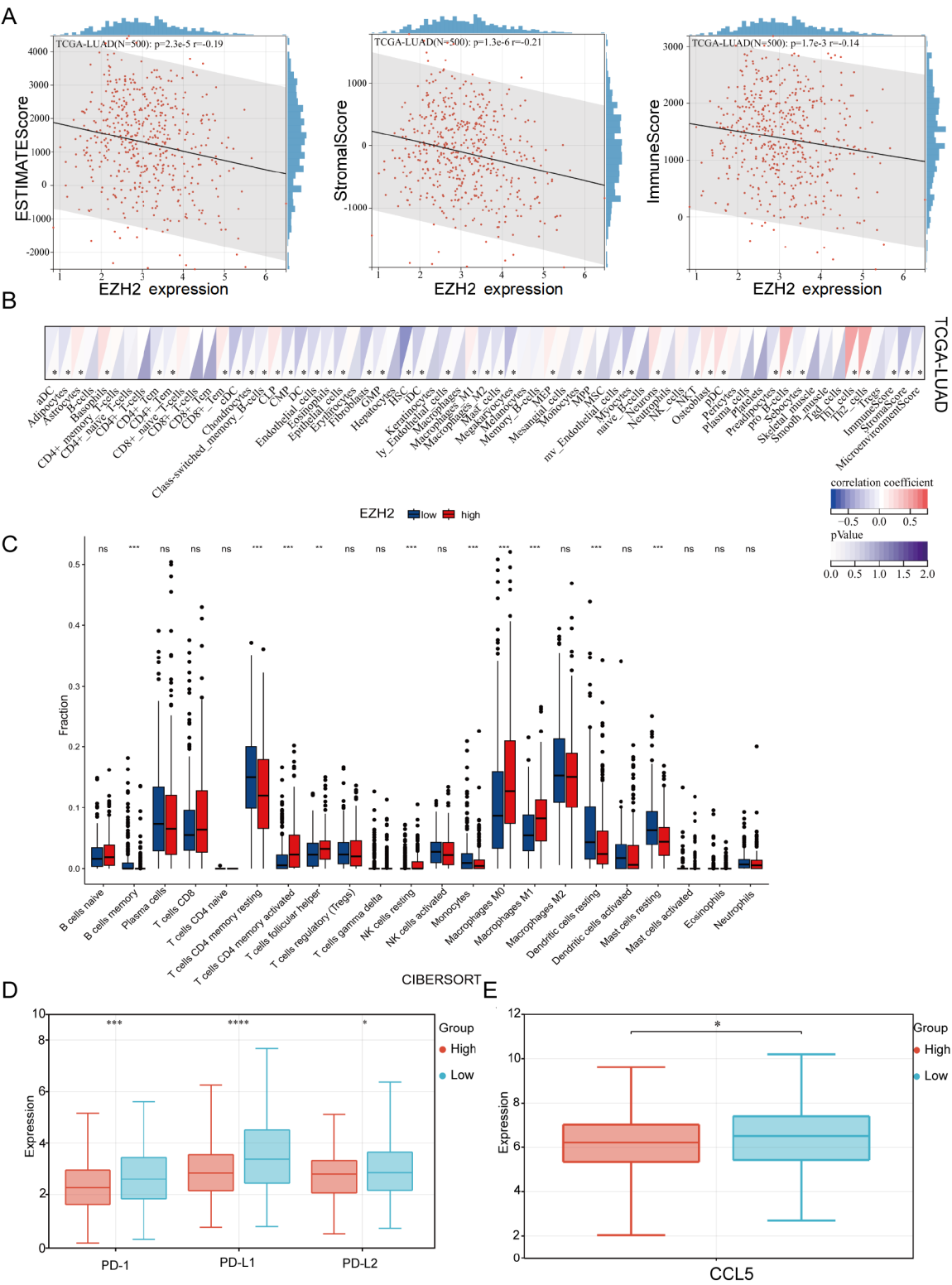


Figure 2. Immune microenvironment analysis of EZH2 in LUAD (A) Immune and stromal infiltration scores (Scatter plot showing the ESTIMATE score, stromal score, and immune score in TCGA LUAD samples). (B) XCELL immune cell enrichment heatmap. (C) CIBERSORT immune cell composition (group comparison). (D) Expressions of PD-1, PD-L1, and PD-L2 in the EZH2 groups. (E) Boxplots of CCL5 expression between EZH2 groups. CCL5 expression was compared between groups using the Wilcoxon rank-sum test.

cells, including B cells, central memory CD8⁺ T cells (TCM), $\gamma\delta$ T cells, natural killer cells (NK cells), and natural killer T cells (NKT cells) (all $P < 0.05$; [Supplementary Figure S3A–C](#)). To explore the impact of EZH2 on the immune microenvironment of LUAD, we performed CIBERSORT analysis on 513 TCGA-LUAD samples to evaluate immune cell composition proportions ([Figure 2C](#) and [Supplementary Figure S3D](#)). In the LM22 immune cell signature matrix, we observed that EZH2 showed significant correlations with multiple immune cell types ([Supplementary Figure S3E,F](#)). These findings suggest that EZH2 may negatively regulate antigen presentation or contribute to immunosuppression within the tumor microenvironment (TME). Analysis of public datasets indicates that higher EZH2 expression is correlated with reduced infiltration of several antitumor immune cell populations, including CD4⁺ and CD8⁺ T cells as well as B cells. Further analysis showed that EZH2 gene expression was negatively correlated with immune checkpoint molecules (PD-1 and PD-L1) and CCL5, suggesting that EZH2 may influence tumor immune evasion by regulating immune cell infiltration ([Figure 2D,E](#)).

Spatial transcriptomics reveals that EZH2 suppresses T-cell recruitment and antigen presentation

For spatial transcriptomic data from GSE189487, we analyzed adenocarcinoma *in situ* (AIS) and minimally invasive adenocarcinoma (MIA) samples ([Figure 3A–D](#)). Major cell types were identified and annotated ([Figure 3E](#)). Spatial transcriptomic analysis revealed a significant difference in EZH2 expression between AIS and MIA ($P < 0.05$), with EZH2 expression being higher in MIA than in AIS. In terms of spatial distribution, EZH2 may be concentrated in the core region of MIA lesions, while showing scattered expression in AIS. CCL5 is highly expressed in the stromal region adjacent to the tumor nest in MIA, whereas in AIS, it is mainly distributed in the peripheral region ([Figure 3F](#)). Combined with the analysis of TCGA RNA-seq data, the results are consistent with the notion that EZH2 regulates the development of lung adenocarcinoma during tumor progression and suggest that CCL5 may mediate the mechanism of tumor immune escape. These differences indicate that the spatial regulation of these genes varies with the pathological stage or subtype of lung adenocarcinoma. In addition, the high expression of CCL5 overlaps with immune cell infiltration regions, suggesting that it may play a role in immune regulation; EZH2 overlaps with tumor cell proliferation regions, implying that it may be involved in cell growth processes. This spatial correlation provides clues for exploring the potential biological functions of these genes in the local microenvironment of lung adenocarcinoma lesions.

Inhibition of EZH2 induces CCL5 production and promotes CD8⁺ T-cell recruitment

Based on bulk RNA sequencing analyses, we identified significant associations between EZH2 and immune cells within the TME. The underlying mechanism may involve a negative correlation between EZH2 and CCL5-mediated chemotaxis. To validate the hypotheses from the bioinformatics findings, we compared the EZH2 protein levels in the normal lung epithelial cell line BEAS-2b with three LUAD cell lines (H1299, PC-9, and A549). Western blotting confirmed higher EZH2 expression in LUAD cell lines ([Figure 4A](#)). CCK-8 cytotoxicity assays demonstrated that the EZH2 small-molecule inhibitor GSK126 suppressed LUAD cell growth ([Figure](#)

[4B,C](#)). To validate the relationship between *EZH2* expression and T-cell infiltration, qRT-PCR was used to measure CCL5 levels in blank controls and GSK126-treated A549, PC9, and H1299 cells. The results showed that *CCL5* expression significantly increased after GSK126 treatment, as confirmed by qRT-PCR ([Figure 4D](#)). PC9, A549, and H1299 cell lines treated with GSK126 exhibited elevated CCL5 levels in total protein. This finding aligns with reports in the literature that CCL5 is associated with T-cell chemotaxis in various cancers [29,30]. Studies in ovarian cancer, head and neck squamous cell carcinoma, and hepatocellular carcinoma have shown that EZH2 inhibition increases the Th1-type chemokines CXCL9 and CXCL10, mediating CD8⁺ T-cell infiltration and chemotaxis [29,30]. However, GSK126 treatment did not significantly increase CXCL9 and CXCL10 mRNA levels in H1299 cells, highlighting tumor heterogeneity and the complexity of EZH2 regulation ([Figure 4E,F](#)).

To further investigate this possibility, we constructed stable *EZH2* shRNA knockdown LUAD cell lines. qRT-PCR and western blot analysis confirmed successful knockdown at both the mRNA and protein levels ([Figure 5A–D](#)). *CCL5* mRNA levels were significantly elevated in *EZH2* shRNA stable cell lines, consistent with GSK126-treated western blot results, suggesting an inverse correlation between EZH2 expression and CCL5 production in tumor cells ([Figure 5E–G](#)). Since chemokine function depends on secretion into the extracellular matrix, we used ELISA analysis of cell culture supernatants to confirm that both GSK126 treatment and *EZH2* knockdown promoted CCL5 secretion, consistent with the qRT-PCR and western blot results ([Figure 5H,I](#)). Transwell assays confirmed that EZH2 inhibition enhanced T-cell chemotaxis mediated by CCL5. GSK126-treated H1299 supernatants exhibited stronger T-cell chemotaxis, which was significantly reduced by CCL5 neutralizing antibodies, confirming CCL5's role in chemotaxis enhancement ([Figure 5J,K](#)).

EZH2 inhibition enhances antigen presentation and T-cell cytotoxicity, independent of PD-L1 levels

In the TME, exhausted CD8⁺ T cells, as well as downregulation of MHC molecules, are common immune evasion mechanisms in cancer. EZH2 inhibitors have been shown to enhance MHC-I antigen presentation in diffuse large B-cell lymphoma (DLBCL) [31] and triple-negative breast cancer (TNBC) by restoring MHC-I expression and potentiating the efficacy of chemotherapy [32]. To explore whether EZH2 affects antigen presentation, qRT-PCR showed that GSK126 treatment or *EZH2* shRNA knockdown increased HLA-A/B/C protein levels, indicating an inverse correlation between EZH2 and HLA-A/B/C expression ([Figure 6A,B](#)). Next, to determine whether this upregulation of MHC-I has a functional consequence on T-cell interactions, we performed a co-culture killing assay. LUAD cells were treated with GSK126 or *EZH2* shRNA and then co-cultured with CD8⁺ T cells from healthy donors. The results showed a significant decrease in surviving tumor cells in the *EZH2*-inhibited groups compared to controls ([Figure 6C,D](#)). In contrast to reports in other cancers, qRT-PCR showed no significant change in *PD-L1* mRNA levels after GSK126 treatment in our study ([Figure 6E](#)), suggesting that the pro-immunogenic effect in our model is primarily mediated through the antigen presentation pathway rather than checkpoint modulation.

Discussion

In recent years, the most landmark research and clinical progress in

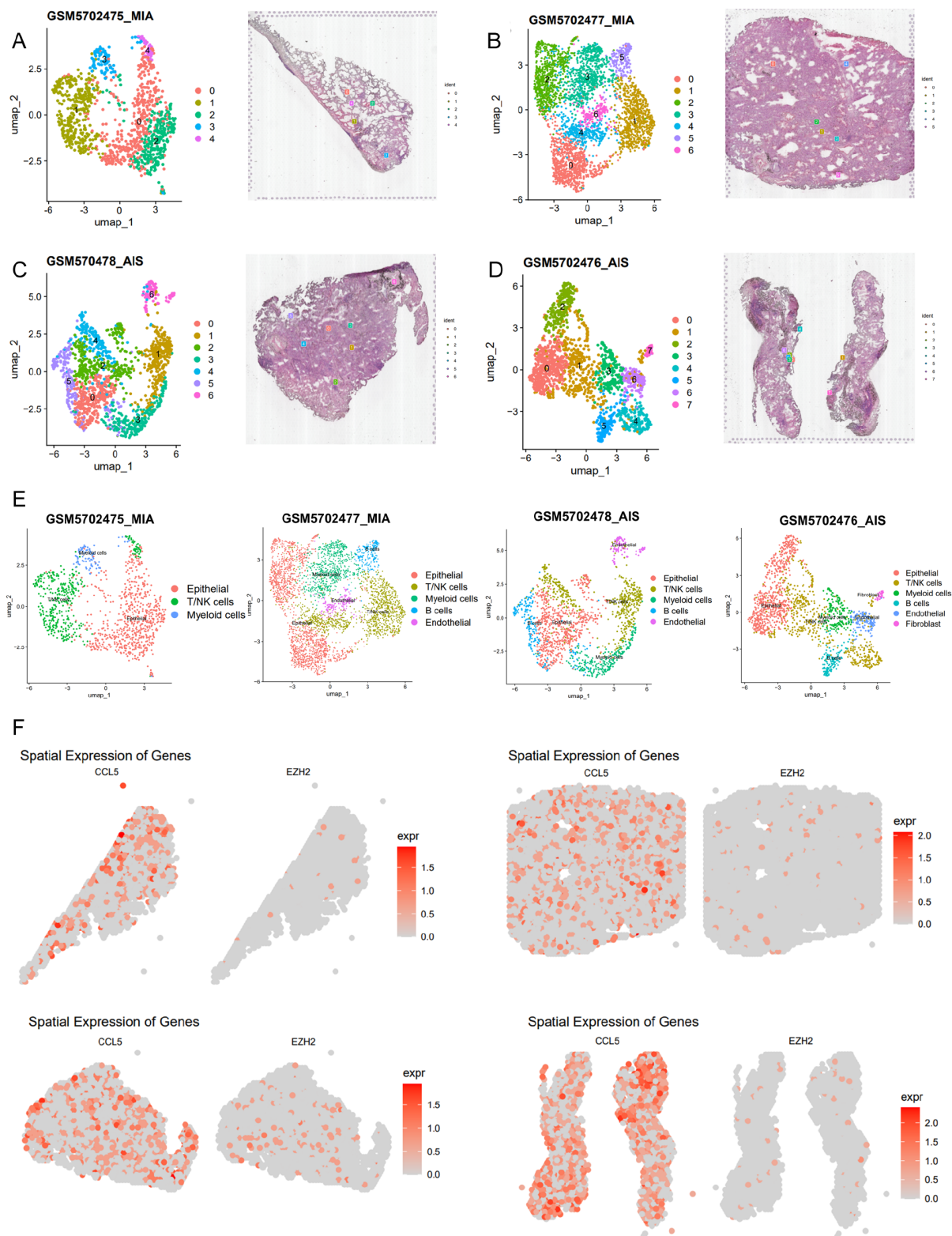


Figure 3. Spatial transcriptomics analysis of AIS and MIA lesions (GSE189487) (A–D) UMAP clustering and tissue localization. UMAP plots show distinct cell clustering in MIA and AIS samples, with different clusters corresponding spatially to histological images. (E) Manually annotated UMAP by cell type. Manual cell type annotation reveals the distribution of various cell types (such as epithelial cells, immune cells, and stromal cells) in different lung adenocarcinoma samples. (F) Spatial projection of EZH2 and CCL5 expression. Genes such as CCL5 and EZH2 exhibit specific spatial expression patterns within tissues. The expression levels of different genes vary in different regions, and their expression patterns differ in MIA, AIS, and other tissues.

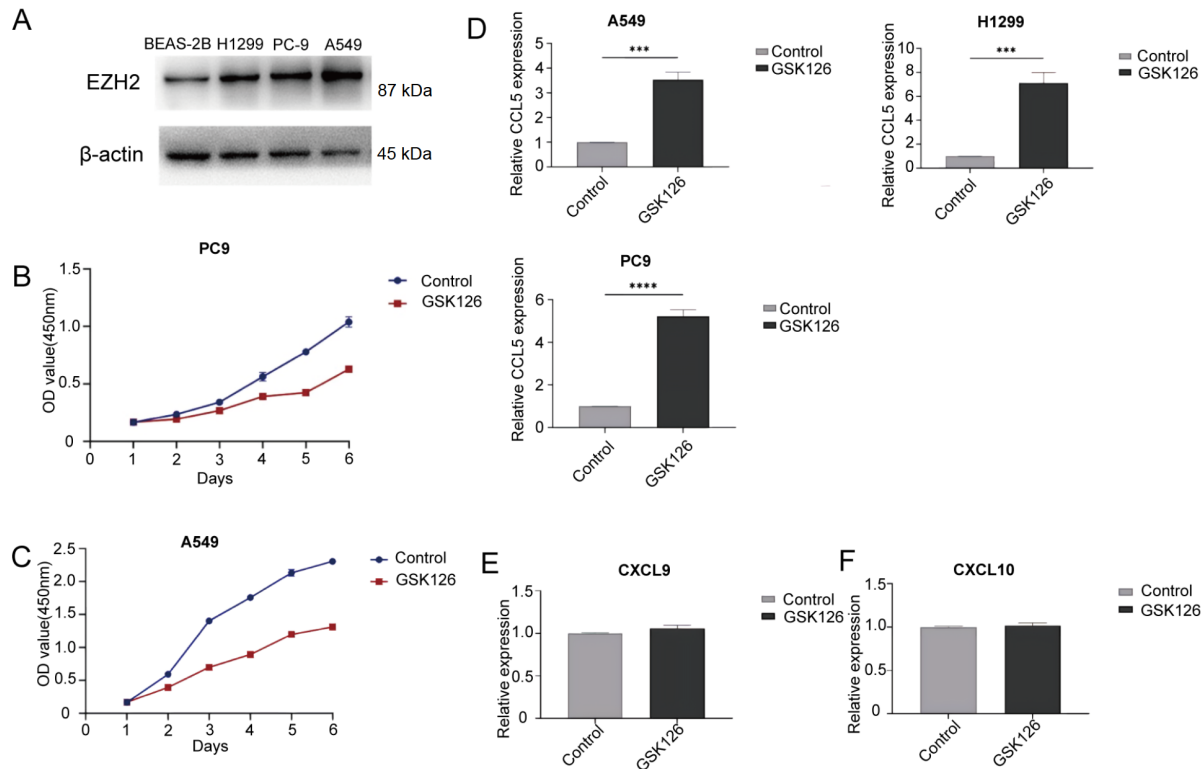


Figure 4. EZH2 inhibition enhances immune-related gene expression in LUAD cell lines (A) Western blot analysis of EZH2 expression. Cell lines: BEAS-2B (normal bronchial epithelial), H1299, PC-9 and A549. (B) CCK-8 assay with an EZH2 inhibitor (GSK126) in PC-9 cells. PC-9 cells were treated with GSK126 (50 μ M) for 1-6 days. Cell viability measured by CCK-8 assay. Data are presented as the mean $\pm$ SD ($n = 3$). (C) CCK-8 assay in A549 cells. (D) qRT-PCR analysis of *CCL5* mRNA after GSK126 treatment. Significant upregulation in all lines ($P < 0.05$, Student's t test). (E,F) qRT-PCR analysis of *CXCL9* (E) and *CXCL10* (F) in H1299 cells.

the field of lung cancer has been the successful application of immunotherapy in the treatment of lung cancer, mainly the use of immune checkpoint inhibitors (ICIs) [33,34]. However, not all patients can benefit from currently approved immune checkpoint inhibitors. In different clinical trial cohorts, the response rate of PD-1 monoclonal antibody used as a single agent is approximately 10%–25% [35]. Ongoing clinical trials are exploring the combination of immune checkpoints with other treatment modalities, including chemotherapy and radiotherapy. To further improve the efficacy of immunotherapy, more in-depth research on the characteristics of the immune microenvironment of non-small cell lung cancer is required to provide strategies for combination therapy with currently used immune checkpoint inhibitors or develop new immunotherapeutic approaches. EZH2 drives lung cancer progression by promoting EMT through epigenetic repression of tumor suppressor genes such as E-cadherin. Its expression is post-transcriptionally upregulated by NOP2-mediated m5C modification of *EZH2* mRNA, which enhances transcript stability in an ALYREF-dependent manner [36]. Clinically, elevated EZH2 expression correlates with poor prognosis in non-small cell lung cancer patients, highlighting its potential as both a prognostic biomarker and therapeutic target [37]. This study focused on revealing the association between the abnormally high expression of EZH2 in LUAD and immune-related molecular pathways and whether targeting EZH2 can enhance the antitumor immune response.

EZH2, as an epigenetic-related molecule, has been reported to

have gain-of-function mutations or overexpression in a variety of tumors, including melanoma, ovarian cancer, and lymphoma. Therefore, EZH2 may become a potential antitumor target [6,38]. In this study, we first aimed to explore the expression of EZH2 in LUAD. Data from the TCGA LUAD case cohort confirmed that, compared with normal tissues, EZH2 was abnormally highly expressed in LUAD tissues, which was consistent with previous literature reports. At the same time, EZH2 was associated with a poor prognosis in patients with LUAD, which also suggests the possible important role of EZH2 in the development of LUAD. Supporting evidence from spatial transcriptomics further demonstrated that EZH2 is markedly upregulated as LUAD progresses from AIS to MIA. Moreover, EZH2 shows a spatially inverse expression pattern relative to *CCL5*. These findings suggest that EZH2 may promote early tumor invasion by suppressing *CCL5*-mediated immune cell recruitment.

The infiltration of immune cells in the tumor microenvironment is an important factor to be considered in tumor immunotherapy. In many tumors, the infiltration of CD8⁺ T cells and DCs in tumor tissues is associated with a better prognosis and the efficacy of immune checkpoint inhibitors [39]. In contrast, the infiltration of regulatory T cells, myeloid-derived suppressor cells, and tumor-associated macrophages in the tumor microenvironment is associated with a poor prognosis and resistance to immunotherapy [40]. To explore the impact of high EZH2 expression on immune-related factors in the tumor microenvironment of LUAD, based on TCGA-

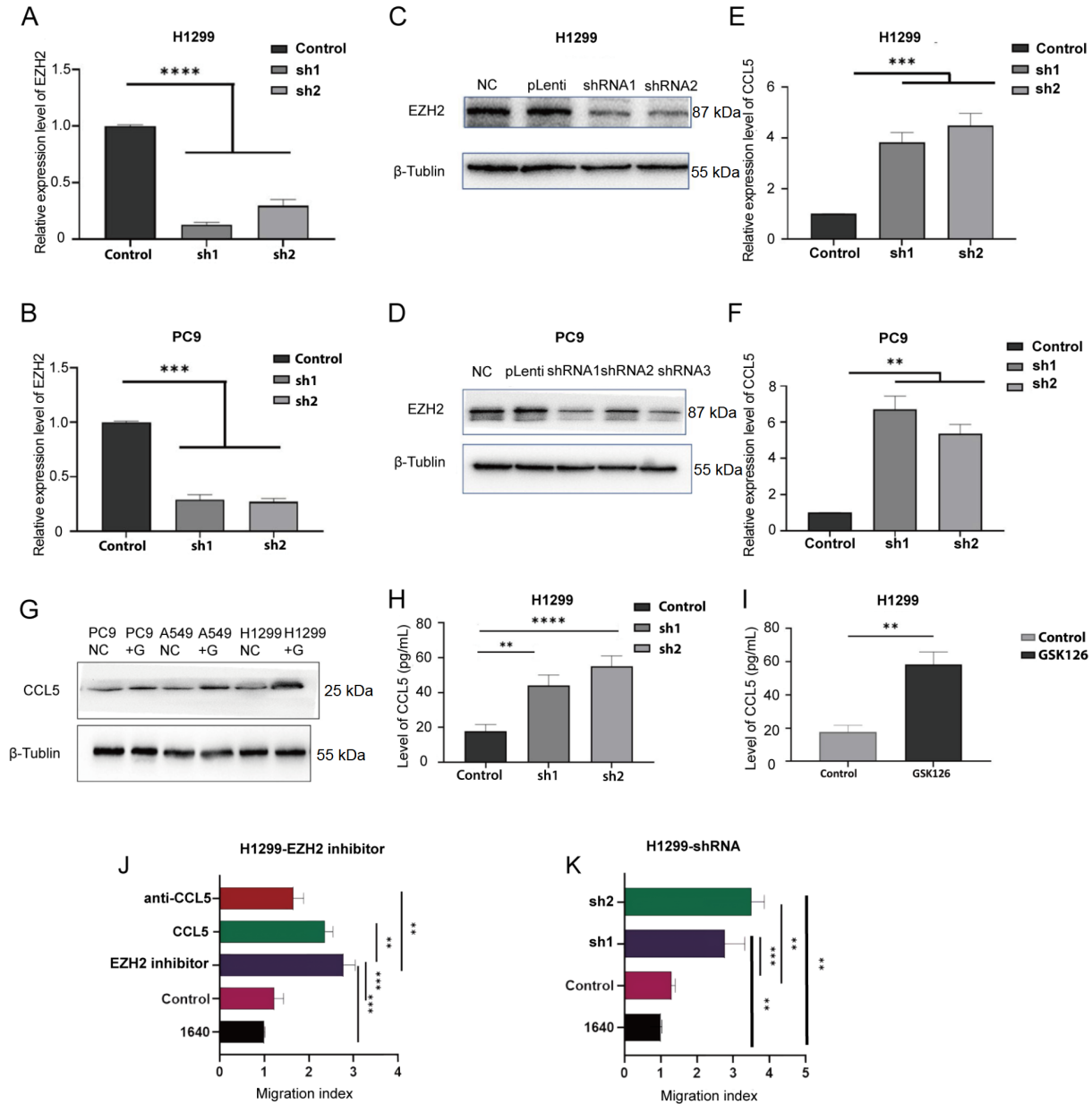


Figure 5. Validation of *EZH2* knockdown and inhibition effects on CCL5 expression and immune cell recruitment (A,B) qRT-PCR validation of *EZH2* shRNA knockdown. (A) H1299: *EZH2* mRNA levels in untransfected control vs two independent shRNA clones (shRNA1 or shRNA2). (B) PC-9: *EZH2* mRNA levels in untransfected control vs two independent shRNA clones (shRNA1 or shRNA2). (C,D) Western blot analysis of *EZH2* knockdown in H1299 (C) and PC-9 (D) cells. (E,F) *CCL5* mRNA upregulation after *EZH2* knockdown. (G) Western blot of CCL5 in PC-9, A549, and H1299 cells after been treated with GSK126 (50 μ M, 48 h). (H,I) ELISA validation of CCL5 secretion. (H) Boxplot showing CCL5 expression in untransfected (control), shRNA1-transfected, and shRNA2-transfected H1299 cells. (I) Boxplot showing CCL5 expression in H1299 cells treated with vehicle control or GSK126 (50 μ M) for 48 h. (J,K) CD8⁺ T-cell migration assay. (J) Experimental groups included: 1640 medium (negative control), recombinant CCL5 (positive control), and H1299 supernatants from untreated, GSK126-treated (50 μ M), or anti-CCL5 antibody-treated cultures. (K) Experimental groups: untransfected control, shRNA1-, or shRNA2-transfected H1299 cells, with 1640 medium as a negative control.

LUAD data, we employed multiple bioinformatic tools for immune infiltration analysis to investigate the immune cell composition and correlations within the TME of LUAD. The results showed that *EZH2* expression was negatively correlated with the infiltration of CD8⁺ T cells, CD4⁺ T cells, and DCs and positively correlated with the infiltration of myeloid-derived suppressor cells (MDSCs).

To reveal the reasons for the correlation between *EZH2* and

immune cell infiltration, considering the important role of chemokines in T-cell migration, we hypothesized that *EZH2* might be related to the synthesis and secretion of tumor cell chemokines. CCL5 is a key immune cell chemokine that mainly mediates the chemotactic migration of T cells to peripheral tissues by acting on the chemokine receptors CCR3, CCR4, and CCR5. The results of qRT-PCR in our study confirmed that after using the *EZH2* inhibitor

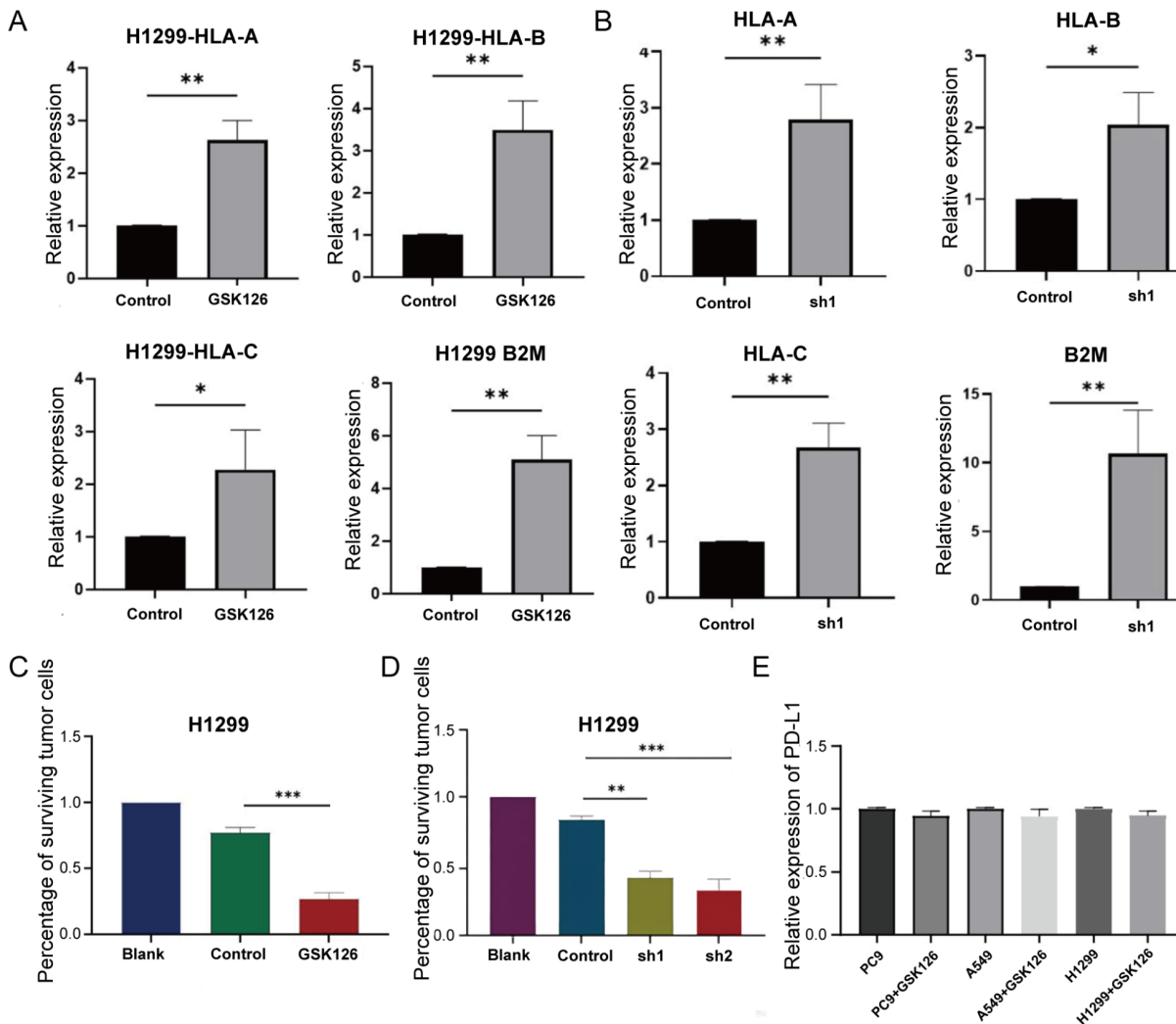


Figure 6. EZH2 inhibition enhances T-cell-mediated cytotoxicity and does not modulate immune checkpoint expression (A,B) qRT-PCR analysis of *HLA class I* and *B2M* mRNA expressions in H1299 cells. (A) Cells were treated with vehicle control or GSK126 (50 μ M) for 48 h. (B) Comparison between control and shRNA-transfected cells. (C,D) Co-culture T-cell killing assays. (C) Experimental groups included: blank, control, and GSK126 (50 μ M, 48 h). (D) Experimental groups included: blank, control, shRNA1, and shRNA2. Statistical comparison: One-way ANOVA with Tukey's post-hoc test. (E) *PD-L1* mRNA expression after GSK126 treatment.

GSK126, the mRNA of *CCL5* in tumor cells increased, and western blot and ELISA confirmed the increase in *CCL5* protein levels and *CCL5* levels in the cell culture supernatant. To determine whether the increased *CCL5* levels could enhance the chemotaxis of $CD8^+$ T cells, we performed Transwell chamber experiments, which demonstrated that *CCL5* could enhance the migration of $CD8^+$ T cells to tumor tissues and played a major role in the increased infiltration of $CD8^+$ T cells induced by EZH2 inhibition. This suggests that EZH2 inhibitors may have a synergistic effect with PD-1 inhibitors and have the potential to increase the efficacy of adoptive cell therapy by increasing the migration and infiltration of $CD8^+$ T cells into tumor tissues.

The presentation of tumor antigens is a key step in triggering the body's antitumor immunity. Tumor-specific $CD8^+$ T cells are activated by antigen-presenting cells (DCs) in regional lymph nodes and migrate to tumor tissues. The specific TCRs expressed on the surface of T cells bind to MHC class I molecule complexes that

present tumor antigens on the surface of tumor cells, inducing tumor cell apoptosis through the secretion of granzyme, perforin, or the Fas/Fas ligand pathway [41]. Downregulation of MHC class I molecule expression by tumor cells is a common strategy for tumor immune evasion [42]. In this study, we found that treating lung cancer cell lines with the EZH2 inhibitor GSK126 could increase the expression of genes related to MHC class I molecule complexes and enhance the level of MHC class I complexes on the surface of tumor cells. *In vitro* co-culture cytotoxicity assays of T cells and tumor cells confirmed that the increased expressions of MHC class I molecules could enhance T-cell-mediated tumor cell killing. However, we have to acknowledge that due to limitations, this assay used T cells from healthy donors rather than patient-specific tumor T cells; thus, the cytotoxic effect may be primarily mediated by an allogeneic response rather than tumor specificity. However, in the autologous environment, the same mechanism could enhance the recognition of tumor cells by the patient's own tumor antigen-

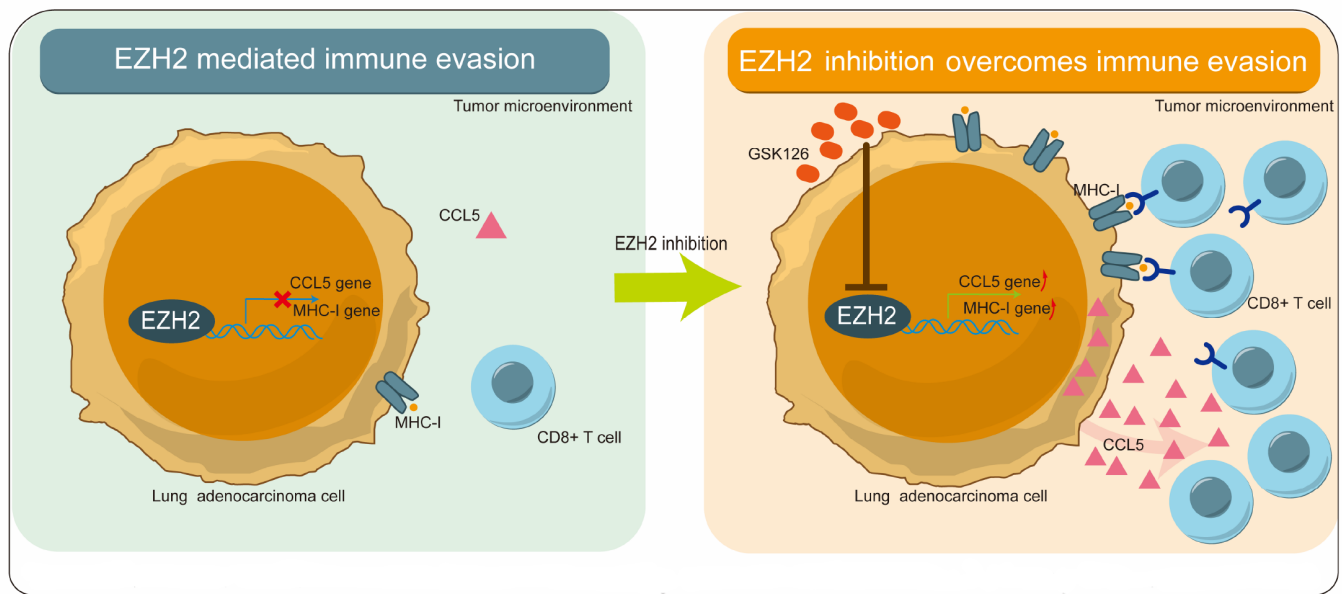


Figure 7. EZH2 inhibitor enhances antitumor immunity via a dual mechanism in lung adenocarcinoma The mechanism figure illustrates that EZH2 inhibitors can regulate MHC class I molecules and CCL5 to mediate antitumor effects. In the left panel, EZH2 suppressed the expression of CCL5 and MHC-I, resulting in reduced CD8⁺ T-cell recruitment and impaired antigen recognition, thereby facilitating immune evasion. In the right panel, EZH2 inhibition (e.g., GSK126) reverses this repression, restoring MHC-I-mediated antigen presentation and CCL5-dependent CD8⁺ T-cell recruitment, together enhancing antitumor immunity.

specific T cells, thereby contributing to a more effective antitumor immune response. Combined with the previous results on T-cell chemotaxis, this further suggests a possible synergistic effect between EZH2 inhibition and immunotherapy.

This study was mainly based on *in vitro* experiments. Considering the limitations of this study and the complexity of tumor immune responses, subsequent research is still needed to verify and further explore the mechanisms. First, we believe that evidence directly from clinical trials is needed to explore the expression level of EZH2 in patients and whether there is a correlation with the response of patients after receiving immunotherapy, which will provide direct evidence for the combined use of EZH2 inhibitors and ICIs. Second, in the tumor microenvironment, there are both positive immune factors, such as CD8⁺ T cells and NK cells, as well as regulatory T cells and MDSCs. Enhancing positive mechanisms may simultaneously activate negative regulatory mechanisms, which may counteract each other, resulting in no significant clinical efficacy. Future research requires direct *in vivo* experimental evidence to confirm that after the action of EZH2 inhibitors, a more intense antitumor immune response is ultimately triggered and to confirm its synergistic effect with immune checkpoint inhibitors. In addition to its known role in T-cell chemotaxis, CCL5 has been reported in the literature to be related to the chemotaxis of DCs. The impact of targeting EZH2 on the antigen presentation pathway also needs to consider the role of antigen-presenting cells. Therefore, *in vivo* experiments, flow cytometry needs to be used to directly analyze the abundance of various immune cells infiltrating the tumor tissue to confirm the mechanisms related to chemokine-mediated immune cell infiltration. In this study, EZH2 inhibitors directly acted on tumor cells, while in animal experiments, the drugs were used systemically, acting not only on tumors but also on

other components, including components related to the immune system. Considering the important role of epigenetic-related mechanisms in the development and function of immune cells, we need to consider whether EZH2 inhibitors will change the functional state of immune cells throughout the body and in the local tumor tissue, thereby affecting the final tumor immune response. In addition to the possibility of combination with immunotherapy, whether secondary resistance to immunotherapy may be related to EZH2-related mechanisms is also a direction for future research.

In summary, our results demonstrate that EZH2 inhibition reverses immunosuppression in LUAD through dual mechanisms: enhancing CCL5-mediated CD8⁺ T-cell chemotaxis and upregulating MHC-I-dependent antigen presentation (Figure 7). Critically, the augmented T-cell cytotoxicity in co-culture assays (Figure 6C,D) aligns with established evidence that EZH2 inhibition epigenetically reprograms T cells to enhance effector functionality [28,43]. This functional reprogramming, combined with increased tumor immunogenicity, provides a mechanistic basis for synergizing with ICB. Our integrated multi-omics approach further illuminates how tumor-intrinsic EZH2 signaling orchestrates immune evasion, offering novel insights for combinational immunotherapy in LUAD.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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