



Single-cell and spatial analysis reveals macrophage-T cell crosstalk in non-small cell lung cancer immunosuppression

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Background: The tumor microenvironment (TME) is instrumental in facilitating immune evasion and promoting tumor progression in non-small cell lung cancer (NSCLC). However, the spatial heterogeneity and molecular interactions between immune and non-immune cells are not yet fully understood. This research amalgamated single-cell RNA sequencing (scRNA-seq) and spatial transcriptomic data to examine the cellular composition, ligand-receptor (LR) interactions, and immunosuppressive mechanisms of NSCLC.

Methods: ScRNA-seq was performed on NSCLC tumors and paired adjacent normal tissues to analyze immune and stromal cell heterogeneity. Spatial transcriptomics was applied to consecutive tissue sections to map the distribution of major cell types and their interactions. Cell clustering, a differential gene expression analysis, and LR interaction modeling were used to identify key immune regulatory pathways. *In vitro* co-culture experiments were performed for functional validation.

Results: A total of 28,496 high-quality single cells were analyzed, and significant differences in immune cell composition between the tumor and normal tissues were found. The NSCLC tumors exhibited a notable increase in tumor-associated macrophages (TAMs) and fibroblasts, as well as a decrease in the presence of cytotoxic T cells and natural killer (NK) cells. Notably, spatial transcriptomics revealed that the TAMs were predominantly localized in the tumor cores, where they interacted with T cells via immunosuppressive LR pairs. The SPP1-CD44 and NECTIN2-TIGIT axes were identified as major immunosuppressive pathways, with the SPP1 secreted by the TAMs contributing to immune evasion. Additionally, the HLA-E-CD8B interactions were significantly upregulated, suggesting a potential mechanism of antigen presentation modulation. The functional validation showed that *SPP1* overexpression (OE) enhanced *PDCD1* and *CD160* expression, further confirming its role in shaping the immunosuppressive microenvironment.

Conclusions: This study established a high-resolution immune atlas of NSCLC, identifying novel macrophage-T cell interactions that drive immune suppression. Our findings suggest that targeting the SPP1-CD44, NECTIN2-TIGIT, and HLA-E-CD8B pathways may improve immune responses in NSCLC. These findings provide novel therapeutic targets and highlight the potential for combination immunotherapies to overcome macrophage-mediated immune evasion.

Keywords: Non-small cell lung cancer (NSCLC); single-cell RNA sequencing (scRNA-seq); tumor microenvironment (TME); spatial transcriptomics

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Introduction

Non-small cell lung cancer (NSCLC) is the most prevalent subtype of lung cancer and the primary cause of cancer-related death worldwide (1). Despite advancements in targeted therapies and immune checkpoint inhibitors, the 5-year survival rate of NSCLC patients remains poor (2). The tumor microenvironment (TME) plays a crucial role in NSCLC progression by shaping immune responses and promoting immune evasion, ultimately enabling tumor growth and metastasis (3). Tumor-associated macrophages (TAMs), regulatory T cells (Tregs), and cancer-associated fibroblasts (CAFs) play key roles in creating an immunosuppressive microenvironment that impedes cytotoxic T lymphocyte (CTL) infiltration and activity, ultimately reducing the effectiveness of immunotherapies (4,5). However, the spatial organization of immune cells and

their molecular interactions in NSCLC are not yet fully understood; thus, more comprehensive analyses need to be conducted to identify novel therapeutic targets.

TAMs, Tregs, and CAFs create an environment that weakens the immune response, making it more difficult for CTLs to enter and work effectively, thereby limiting the efficacy of immunotherapies (6). Previous studies have shown that NSCLC tumors exhibit high heterogeneity in immune and stromal cell composition, with TAMs displaying a M2-like immunosuppressive phenotype that fosters tumor progression (7,8). Extensive research has been conducted on immune checkpoint pathways such as *PD-1* (programmed cell death protein 1), *CTLA-4* (cytotoxic T-lymphocyte-associated protein 4), and *TIGIT* (T cell immunoreceptor with Ig and ITIM domains); however, the specific ligand-receptor (LR) interactions mediating macrophage-T cell crosstalk in NSCLC are unclear (9). Further, conventional single-cell RNA sequencing (scRNA-seq) techniques lack spatial resolution, which poses challenges in identifying the exact positioning and interactions of immune cells in the three-dimensional structure of tumors.

Recent advancements in spatial transcriptomics have provided a powerful approach for mapping cellular interactions in the TME while preserving tissue architecture (10). This technique allows for the identification of immune exclusion mechanisms, where T cells are physically restricted from infiltrating tumor regions, a phenomenon frequently observed in solid tumors, including NSCLC (11). TAMs are pivotal in immune exclusion and are particularly notable for their frequent localization at the tumor-stroma interface. In this region, they engage in LR interactions that suppress T-cell activity and facilitate immune evasion (12,13). However, the specific molecular pathways mediating these interactions in NSCLC are poorly defined.

In this study, we integrated scRNA-seq and spatial transcriptomics data to construct a high-resolution immune atlas of NSCLC, systematically characterizing the cellular composition, spatial organization, and LR interactions that define the immunosuppressive TME. We hypothesize that TAMs play a central role in orchestrating T-cell dysfunction via specific LR interactions. By combining computational analyses with *in vitro* functional validation, we aimed to identify key macrophage-T cell communication pathways, with a particular focus on SPP1-CD44, NECTIN2-TIGIT, and HLA-E-CD8B interactions. These results provide insights into the mechanisms of the immune system

Highlight box

Key findings

- This study conducted a comprehensive single-cell and spatial transcriptomic analysis of the non-small cell lung cancer (NSCLC) tumor microenvironment, and identified macrophage-T cell crosstalk as a major driver of immune suppression. Tumor-associated macrophages (TAMs) were shown to be enriched in tumor cores and interact with T cells via immunosuppressive ligand-receptor pairs, notably including SPP1-CD44, NECTIN2-TIGIT, and HLA-E-CD8B. Functional assays confirmed that macrophage-derived *SPP1* enhances *PDCD1* and *CD160* expression, reinforcing T-cell exhaustion. Our findings highlight key immunosuppressive pathways that may serve as novel therapeutic targets to enhance anti-tumor immunity in NSCLC.

What is known, and what is new?

- TAM and T-cell exhaustion contribute to immune suppression in NSCLC.
- This study identified spatially-resolved SPP1-CD44 and NECTIN2-TIGIT interactions as key immunosuppressive axes between TAMs and T cells.

What is the implication, and what should change now?

- The identification of macrophage-driven immunosuppressive pathways suggests that TAMs are not merely passive bystanders but active architects of immune escape in NSCLC. Targeting SPP1-CD44 and NECTIN2-TIGIT interactions may restore T-cell function and enhance responses to checkpoint blockade therapy. Therapeutic strategies should consider combination regimens that disrupt macrophage-T cell crosstalk, particularly in immune-excluded tumors. This study supports the future clinical development of macrophage-targeted immunomodulators as adjuncts to existing immune checkpoint inhibitors.

in NSCLC and could lead to the development of novel treatments that improve immunotherapy by reducing the immune suppression caused by macrophages. We present this article in accordance with the MDAR reporting checklist (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-2025-912/rc>).

Methods

Data download, quality control, data filtering, and data normalization

The scRNA-seq data (GSE198099) were obtained from the Gene Expression Omnibus (GEO) database. The spatial transcriptomics data were obtained from the BioStudies repository (available at: <https://www.ebi.ac.uk/biostudies/>) using the accession number: E-MTAB-13530. The data can be found in the article and supplementary information, or can be obtained from the authors on reasonable request. All data analyzed in this study were obtained from publicly available repositories (GEO and BioStudies). Therefore, ethical approval and informed consent were not required. The study was conducted in accordance with the Declaration of Helsinki and its subsequent amendments.

Seurat 4.0 was used to normalize the expression matrices using the “NormalizeData” and “ScaleData” functions. Subsequently, the “FindVariable” function was used to determine the 4000 most variable genes and to perform a principal component analysis. Using the “FindClusters” function, the first 50 principal components were applied at a resolution of 1.0 to create 24 distinct cell clusters. The clusters with the same cell type were aggregated for further analysis. The final outcomes were carefully reviewed to confirm their accuracy and were visualized using Uniform Manifold Approximation and Projection (UMAP). The main types of cells were chosen by first examining the differentially expressed genes (DEGs) in each group, and then reviewing the relevant literature. The “FindMarkers” tool in Seurat was used to identify the DEGs.

Tumor and adjacent tissue classification based on scRNA-seq expression

A score for the tumor and adjacent tissues was established for each NSCLC patient. This score was calculated based on the average proportion of tumor cells expressing marker genes indicative of either the tumor tissue [epithelial cell

adhesion molecule (*EPCAM*), *KRT19*, *MKI67*, or *CD44*] or adjacent tissue (*SFTPC*, *AGER*, *COL1A1*, or *CD68*). Each patient was assigned to the molecular subtype associated with the highest calculated score. All the subsequent groupings were determined based on the final patient classification.

Comparing the number of different cell types in various samples

To evaluate the abundance of different cell types, the amount of each cell type in individual patients and in larger cell groups in the samples that were not enriched for CD235⁺ cells was measured. The Wilcoxon rank-sum test was used to compare the proportions of different types of cells in the tumor and adjacent tissues. To account for multiple testing, a two-sided Bonferroni correction was used for each group examined. Pearson's product-moment correlation coefficients were used to analyze the associations between the relative abundances of different immune cell types.

Cell-type annotation

The “SingleR” package was employed for cell annotation purposes. The identified clusters were annotated at both the cluster and single-cell levels. Major immune and stromal cell populations were defined based on canonical marker genes (e.g., *CD3E/CD4* for CD4⁺ T cells, *CD3E/GZMK* for CD8⁺ T cells, *LYZ/CD14* for myeloid cells, *COL1A1/DCN* for fibroblasts, and *EPCAM/AGER* for epithelial cells), as detailed in the Results section. Annotation was further validated by differential expression analysis and comparison with published single-cell lung atlases.

Gene Ontology (GO) analysis

The GO enrichment analysis was performed using the “clusterProfiler” package in R. The “FindMarkers” function in Seurat enabled the identification of DEGs with a significance level of 0.05. The “clusterProfiler” package was subsequently used to conduct the functional enrichment analysis of the identified DEGs. The GO terms were sorted into the following three categories: biological processes, cellular components, and molecular functions. The enrichment analysis results were displayed in dot plots and bar charts created in R.

CellChat

CellChat (version 2.0) was used to examine the ways in which the cells communicate with each other using the scRNA-seq data. The normalized and batch-corrected expression matrix was input into CellChat, with the cell types annotated based on known marker genes. Genes encoding known LR pairs were filtered using the CellChatDB. Communication networks were inferred by computing the interaction probabilities between the cell types, and statistical significance was assessed by permutation testing. The key signaling pathways were identified and analyzed using centrality measures and network visualization.

Spatial cell typing with cell2location

Cell2location was used to analyze the types of cells in each specific place (spot). As the scRNA-seq reference dataset comprised both tumor and normal samples, deconvolution was performed independently for each tissue type. Tumor-specific annotations were used to estimate the spatial cell composition in the tumor sections, while normal annotations were used for the adjacent tissue datasets. To determine cell-type abundance in the tumor and background regions, the q05 cell abundance values inferred by cell2location were summed across spots that met the quality control criteria. The composition of cell types was found by normalizing the abundance of each type to the total abundance of all cell types. In the tumor sections, we assessed the correlation distance of cell-type composition across qualifying spots, applied hierarchical clustering with full linkage, and presented the results as a dendrogram. In this process, annotated scRNA-seq clusters were used as the reference matrix, allowing cell2location to directly map each subpopulation onto Visium spots. To validate this mapping, we compared the inferred cell-type abundances with the expression patterns of canonical marker genes (e.g., CD3E/CD4 for T cells, LYZ/CD14 for macrophages), confirming the spatial consistency of TAM enrichment in tumor cores.

LR colocalization analysis

To analyze the expression of LR pairs using the 10× Visium platform, we first binarized the expression levels of each gene in the LR pairs across spots that met the quality control standards. A gene was labelled as expressed in a spot if its expected amount, measured by cell2location,

was higher than the average amount for that gene in that specific tissue section. Tumor and background spots from the same patient were collected, noting whether both genes in a LR pair were either active together or not active at all. The χ^2 test was used to examine the contingency tables. For multiple comparisons, a strict Bonferroni correction was performed on the P values for all the LR pairs that were found to be common in tumors. A LR pair was considered significantly enriched in tumors if the Bonferroni-adjusted P value was less than 0.05 in at least four cases.

Cell culture

The A549 cells (purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were grown in F12/K (Kaighn's Modification of Ham's F-12) medium with 10% fetal bovine serum (FBS) to which 100 units/mL of penicillin and 100 µg/mL of streptomycin was added. The THP-1 cells (obtained from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were turned into macrophages via treatment with 100 ng/mL of phorbol 12-myristate 13-acetate (PMA) for 24 hours, and were then kept in PMA-free medium for 48 hours. The CD8⁺ T cells (purchased from Shanghai Aoneng Biotech Co., Ltd., Shanghai, China) were isolated using anti-CD8 magnetic beads, and then activated with anti-CD3 and anti-CD28 antibodies in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS and 100 U/mL of interleukin-2 (IL-2) for 3 days.

Construction of gene mutations

For the *SPP1* knockout (KO), CRISPR-Cas9 technology was employed using single-guide RNA (sgRNAs) targeting the *SPP1* gene, with non-targeting sgRNAs serving as a control (sg1: GCGGTTTCACAGGGACTACC, and sg2: GCTGGATGACCTCAGAAGAC). The sgRNAs were designed and cloned into a lentiviral vector, and then transduced into the cells. The stable KO clones were selected using puromycin.

For the *SPP1* overexpression (OE), the full-length *SPP1* complementary DNA (cDNA) was cloned into a lentiviral expression vector. The THP-1 cells were transduced with the lentivirus, and the stably transduced cells were selected using hygromycin. Transduction efficiency and gene editing were confirmed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) to verify the *SPP1* expression levels (Figure S1).

Direct co-culture

For the direct co-culture, A549 cells were seeded in six-well plates at a density of 5×10^4 cells/well, and left to adhere for 24 hours. Macrophages and CD8⁺ T cells were added to the wells at a 1:1:1 ratio with the A549 cells. The co-cultures were kept in RPMI-1640 medium to which 10% FBS and 100 U/mL IL-2 were added. After 72 hours, the cells were collected for more research.

RNA extraction and qRT-PCR

Total RNA was extracted from the cultured cells using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. The RNA was reverse-transcribed into cDNA using HiScript[®] II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China), a commercial reverse transcription kit that includes genomic DNA removal. RT-qPCR was performed using qPCR with SYBR Green dye-based detection, utilizing the Hieff UNICON[®] qPCR SYBR Green MasterMix (Yeasten, Shanghai, China) on a QuantStudio[™] Dx Real-Time PCR machine (Applied Biosystems, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control for normalization, and the primers for qRT-PCR are detailed in Table S1.

Western blot

The cells were lysed with radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors to allow protein extraction, and then centrifuged at 12,000 rpm for 15 minutes. The supernatant was then collected and quantified using a bicinchoninic acid (BCA) assay kit (Beyotime, China). The protein expression levels of the target gene were assessed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed using the Bio-Rad gel electrophoresis and imaging system (Bio-Rad Laboratories, USA). The antibodies used for the Western blot analysis included anti-GAPDH (Abcam, ab8245), anti-CD68 (Abcam, ab955), anti-PD1 (Abcam, ab237728), and anti-CD160 (Abcam, ab202845).

Statistical analysis

The statistical analysis was performed using R (version 4.42) and GraphPad Prism (version 10). The data are presented as the mean plus or minus the standard deviation. The

Student's *t*-test or a one-way analysis of variance was used to compare the different groups. A *P* value less than 0.05 was considered statistically significant.

Results

ScRNA-seq and spatial atlas of NSCLC samples

The study sought to examine differences between immune and non-immune cells, and their organization in both NSCLC and adjacent normal tissues. Two groups of tumor and paired normal tissues (background tissues) were retrieved from the GEO database for scRNA-seq. In addition, a 10× Visium spatial transcriptomics analysis was performed on consecutive 10-μm sections derived from eight human NSCLC tumors (20 sections), eight adjacent non-involved lung tissues (16 sections), and the lung tissues of two healthy donors (4 sections); the data were deposited in the BioStudies repository (Figure 1A).

Quality control, data filtering, and data normalization

The biopsy samples of two NSCLC patients with varied histological and molecular characteristics were analyzed using scRNA-seq. After following stringent quality control and filtering criteria, a total of 28,496 high-quality cells were retained for analysis. These cells met the predefined thresholds for key quality metrics, including the number of detected genes per cell ranging from 200 to 8,000, total RNA molecule counts (UMIs) between 500 and 40,000, and mitochondrial RNA content below 20% (Figure 1B). Genes with a higher standard deviation were chosen for further analysis, as they reflected significant variability across different cells. In total, 1,500 genes with elevated standardized variance were identified. Figure 1C shows the top 10 genes with the most variation.

Tissue-specific marker expression highlights immune modulation in the TME

Through the UMAP visualization and clustering analysis, the cell-type annotation (Figure 1D, cell_type_4) identified the major cell groups, including epithelial cells (Epi_01_NREP, Epi_02_SLPI, Epi_03_cycling, Epi_04_AGER, and Epi_05_NAPSA), lymphoid cells [B cells, natural killer (NK) cells, T01_CD8_GZMK, T02_CD4_S100A4, T03_CD8_TIGIT, T04_CD8_GZMH, T05_CD8_CCL5, T06_CD4_RP, and T07_CD4_IL7R], myeloid cells (M01_Macro_

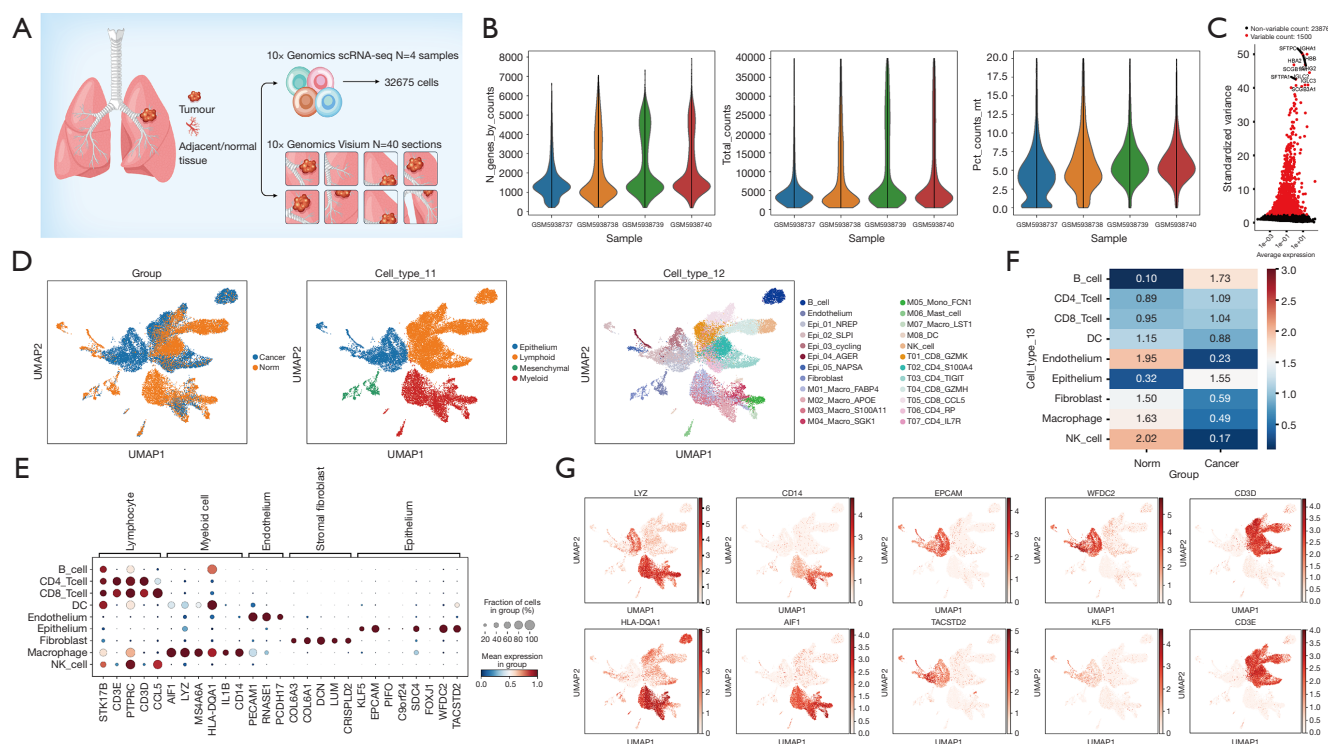


Figure 1 Single-cell transcriptomics revealed tissue-specific markers of immune modulation in the tumor microenvironment. (A) Overview of single-cell RNA sequencing (N=4, 32,675 cells) and spatial transcriptomics (N=40 sections) from tumor, adjacent, and normal lung tissues. (B) Quality-control metrics showing the sequencing depth, UMI counts, and mitochondrial gene percentages across samples. (C) Variance plot showing gene expression diversity in non-small cell lung cancer; the red dots indicate high variability genes, while the black dots indicate low variability genes. (D) UMAP clustering showing cell populations. (E) Dot plot of representative genes used for broad cell-type annotations in tumor samples. (F) Heatmap of cell-type proportions showing the differences between tumor and normal tissues. (G) UMAP maps showing the spatial distribution of the key genes involved in immune suppression and tumor progression. DC, dendritic cell; NK, natural killer; UMAP, Uniform Manifold Approximation and Projection; UMI, unique molecular identifier.

FABP4, M02_Macro_APOE, M03_Macro_S100A11, M04_Macro_SKAI, M05_Mono_FCN1, M06_Mast_cell, M07_Macro_LST1, and M08_DC), and mesenchymal cells (fibroblasts and endothelial cells), which provided a finer resolution of the cellular composition.

To determine if the labels for the cell types were correct, the expression patterns of well-established marker genes across the identified clusters were examined. As expected, the immune cells showed distinct marker profiles, including CD4⁺ T cells with *CD3E* and *CD4*, and CD8⁺ T cells with *CD3E* and *GZMK*. The myeloid cells, including macrophages and dendritic cells (DCs), expressed markers such as *LYZ*, *CD14*, and *HLA-DOA*. The stromal fibroblasts were identified by their high levels of *COL6A1* and *DCN*, while the endothelial cells were identified by the presence of *PECAM1* and *RNASE1*. The epithelial subsets were

delineated by *EPCAM* and cell-specific markers, including *AGER* for alveolar epithelial cells (Figure 1E). In summary, we annotated the major cell populations based on canonical markers: CD3E, CD3D, CD4, and GZMK for T-cell subsets; CD79A and CD79B for B cells; NCAM1 and GNLY for NK cells; LYZ, CD14, HLA-DOA, and AIF1 for myeloid cells; COL1A1, COL6A1, and DCN for fibroblasts; PECAM1 (CD31) and VWF for endothelial cells; and EPCAM, KRT19, and AGER for epithelial subsets.

In addition, the comparison of the normal and cancer tissues revealed significant differences in cell-type composition. The epithelial cells were markedly enriched in the cancer samples compared to the normal tissues, reflecting epithelial proliferation in the TME. Conversely, the NK cells were predominantly observed in the normal

tissues, suggesting potential immune suppression in the cancer tissues. The fibroblasts and endothelial cells also exhibited differential abundance, such that the fibroblasts were more prominent in the cancer tissues, which likely contribute to the desmoplastic response in tumors (Figure 1F). A large decrease was observed in the levels of the markers for epithelial cells in the tumor group compared to the normal group. Conversely, the macrophage markers were notably upregulated in the tumor group, indicating enhanced macrophage infiltration in the TME. Specifically, representative macrophage markers, including *LYZ*, *CD14*, *HLA-DQA1*, and *AIF1*, exhibited a pronounced increase in expression in the tumor tissues. Conversely, the expression of epithelial markers such as *EPCAM*, *AGER*, *TACSTD2*, *WFDC2*, and *KLF5* was decreased, further corroborating the depletion of epithelial cells in the TME (Figure 1G).

Interestingly, while T-cell marker expression (*CD3E* and *CD3D*) was only modestly increased in the tumor tissues, more prominent changes were observed in terms of the epithelial and macrophage markers. The slight elevation in the T-cell markers suggests potential immune surveillance activity and may indicate limited T-cell infiltration or activation in the area around the tumor.

Epithelial and mesenchymal cell changes in the TME

Cell clustering was performed to investigate cellular heterogeneity in both the tumor and normal tissue samples. The clustering results for the epithelial cells are shown in Figure 2A and Figure S2A, and different epithelial subtypes, including Epi_01_NKRP, Epi_02_SLT, Epi_03_Cycling, Epi_04_AGER, and Epi_05_NAPSA, were identified. The associated expression levels across these subtypes are presented in Figure 2B, and major changes in the levels of these markers between the healthy and cancerous groups were observed. Specifically, Epi_01_NKRP and Epi_02_SLT were more highly expressed in the normal group, while Epi_03_Cycling was more lowly expressed in the tumor group.

Conversely, the clustering of the mesenchymal cells (Figure 2C and Figure S2B) revealed distinct populations of endothelial cells and fibroblasts. A study of the two groups (Figure 2D) showed that the tumor group had fewer endothelial cell markers and more fibroblast markers, suggesting that tumor progression is associated with enhanced fibroblast infiltration and a loss of endothelial cells.

To explore the molecular mechanisms that contribute

to these alterations, a GO enrichment analysis of the epithelial and mesenchymal cell populations was conducted. The epithelial cell results (Figure 2E, top panels) showed enrichment in processes related to membrane signaling, the cytokine response, and the cellular response to external stimuli in the normal tissues, while the tumor tissues showed an increase in activities linked to cell-cycle control and the addition of phosphate groups to proteins. In relation to the mesenchymal cells, the lower panels of Figure 2E indicate that the fibroblasts in the tumor group were enriched in pathways associated with collagen formation, extracellular matrix (ECM) organization, and wound healing. While in the tumor group, the endothelial cells showed less activity in the processes related to angiogenesis and signaling from vascular endothelial growth factor.

Macrophage and T-cell dynamics in the TME

Cell clustering was performed to explore the heterogeneity of macrophages and T cells in the tumor and normal tissues. The macrophages were classified into distinct subtypes, including M01_Macro_FABP4, M02_Macro_APOE, M03_Macro_S100A11, M04_Macro_SGK1, M05_Mono_PCNA, M07_Macro_LST1, and M08_DC (Figure 3A and Figure S2C). The ratio of observed to expected (RO/E) analysis (Figure 3B) revealed that M01_Macro_FABP4 was more highly expressed in the normal group, while M02_Macro_APOE and M08_DC were significantly enriched in the tumor group, suggesting macrophage subtype reprogramming in the tumor. The marker analysis (Figure 3C) revealed that the TAMs expressed elevated levels of antigen presentation molecules (*HLA-DRA* and *CD74*) and pro-inflammatory markers (*IL-1B*), indicating their role in promoting inflammation and immune modulation. Additionally, metabolic markers such as *APOE* were upregulated in the TAMs, reflecting metabolic reprogramming to support tumor progression. Among these TAM subsets, APOE⁺ and SPP1⁺ macrophages were identified as the predominant sources of SPP1 expression and were enriched in tumor cores, suggesting that immunosuppressive signaling originates from specific TAM subtypes rather than the entire TAM population (Figure 3B,3C).

The T cells were divided into subtypes, including T01_CD8_GZMK, T02_CD4_S100A4, T03_CD4_TIGIT, T04_CD8_GZMB, T05_CD8_CCL5, T06_CD4_RP, and T07_CD4_IL7R (Figure 3D and Figure S2D). The RO/E analysis showed a reduction in cytotoxic T cells (Figure 3E),

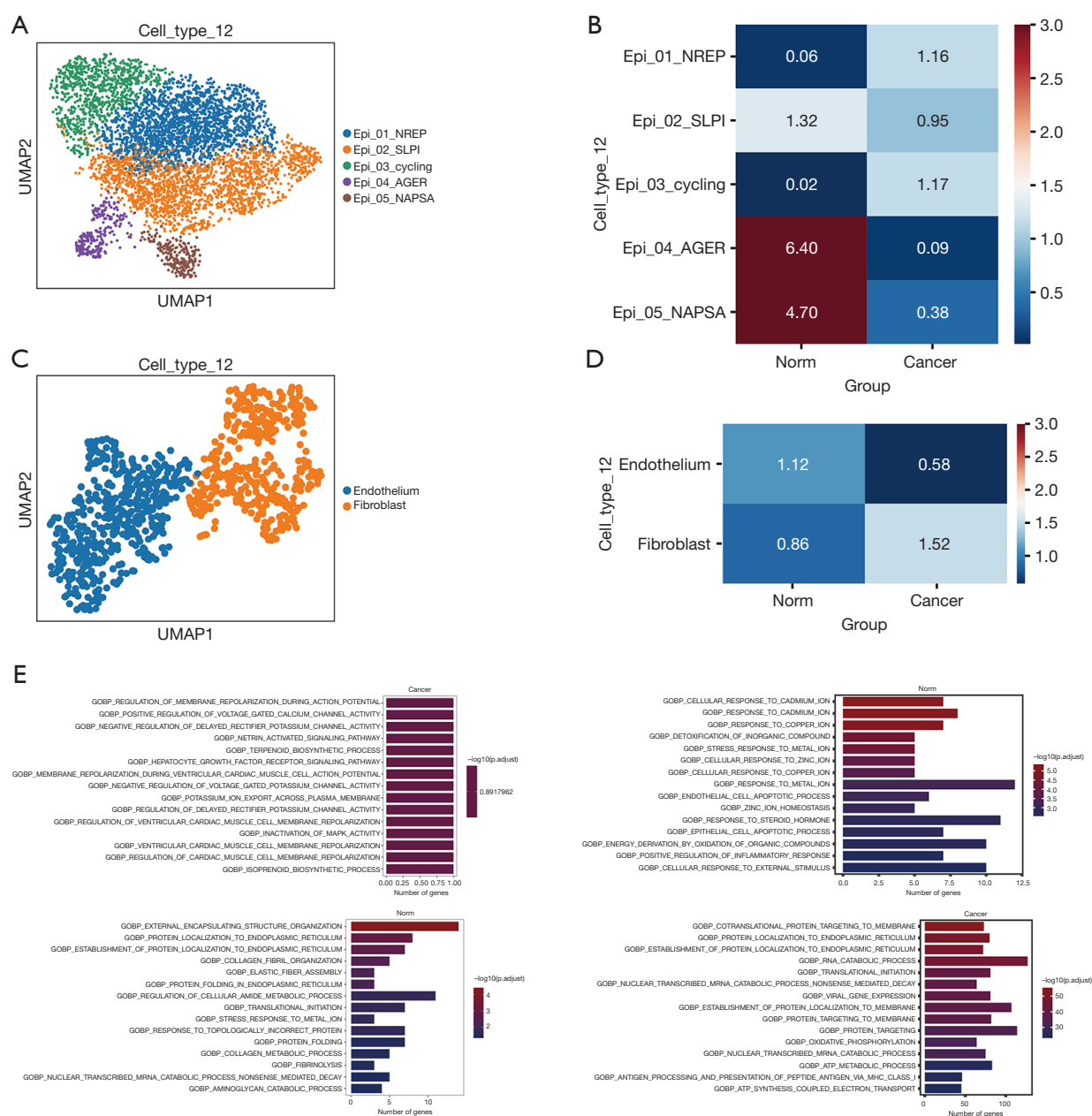


Figure 2 Epithelial and mesenchymal cell changes in the tumor microenvironment. (A) UMAP clustering showing epithelial subpopulations in normal and tumor tissues. (B) Heatmap showing epithelial subtype proportions and their changes in normal and tumor tissues. (C) UMAP clustering showing the mesenchymal subpopulations in normal and tumor tissues. (D) Heatmap showing mesenchymal subtype proportions and their changes in normal and tumor tissues. (E) Functional enrichment analysis of endothelial and fibroblast pathways. UMAP, Uniform Manifold Approximation and Projection.

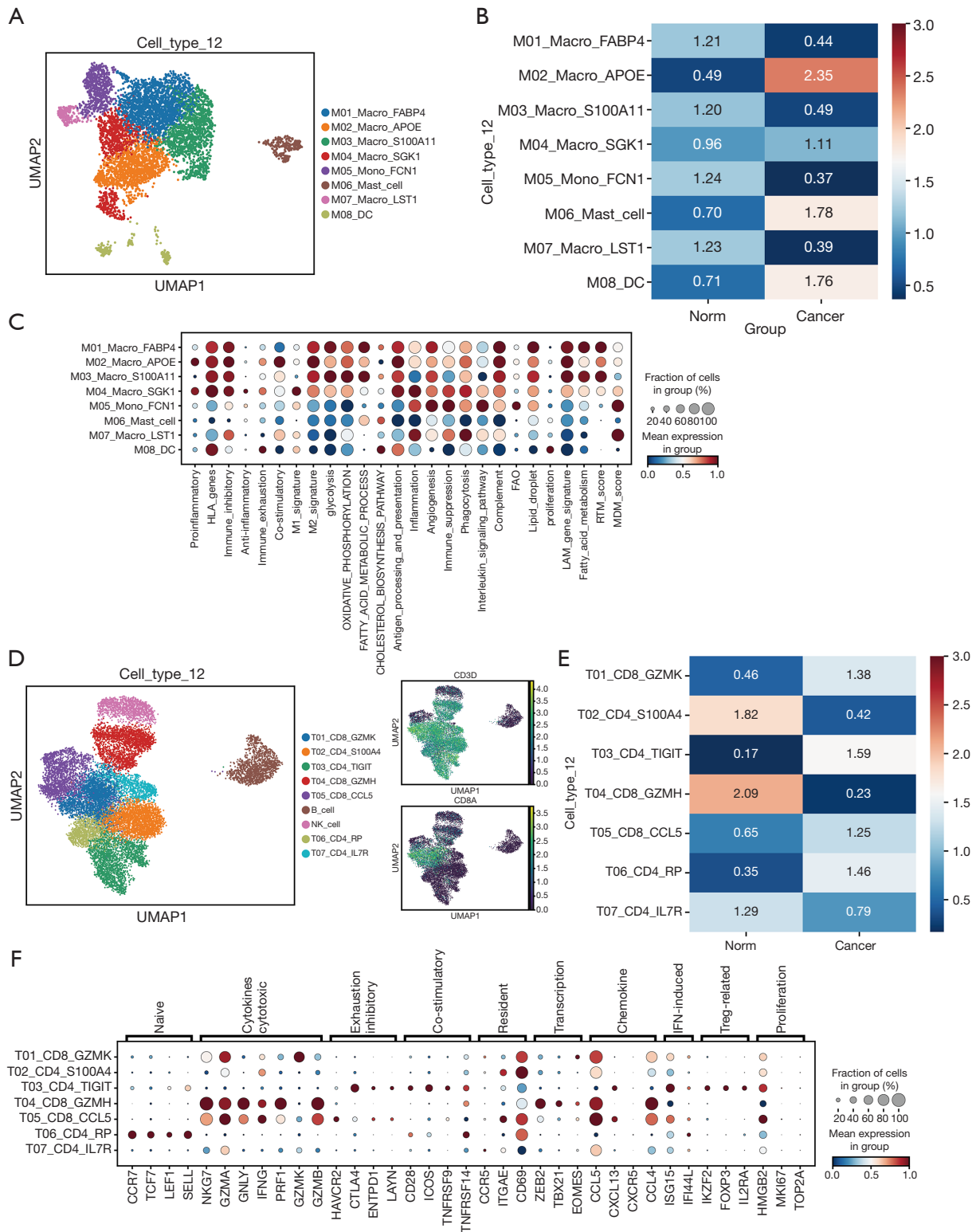


Figure 3 Macrophage and T-cell dynamics in the tumor microenvironment. (A) UMAP clustering showing macrophage subpopulations in normal and tumor tissues. (B) Heatmap showing macrophage subtype enrichment across conditions. (C) Dot plot showing macrophage marker gene expression in tumor samples. (D) UMAP plot showing T-cell subpopulations. (E) Heatmap showing T-cell subtype enrichment across tissues. (F) Dot plot showing T-cell marker gene expression. UMAP, Uniform Manifold Approximation and Projection.

such as T04_CD8_GZMB, with decreased expression of markers *GZMB* and *PRF1*, while Tregs, such as T03_CD4_TIGIT, were enriched in the tumor group, and the expression levels of the immune checkpoint markers *TIGIT* and *CTLA4* were elevated. The marker analysis (Figure 3F) revealed that the tumor-associated T cells exhibited a shift toward an immune-suppressive phenotype, with lower levels of pro-inflammatory substances [e.g., interferon gamma (IFNG) and tumor necrosis factor (TNF)] and higher signs of T-cell fatigue and control. Together, these findings highlight significant macrophage-T cell rearrangement in the areas around tumors, where macrophages adopt pro-tumor roles and T cells lose their cytotoxic functionality, facilitating immune evasion by the tumor.

Macrophage-T cell interactions in cancer and normal tissues

To examine how macrophages and T cells communicate with each other, we analyzed the relative and absolute information flow of LR interactions in normal and cancer tissues. The left panel of Figure S3A shows the relative information flow of different signaling pathways, while the right panel shows the absolute information flow in both tissues. Our results revealed that the macrophage-T cell interactions were significantly altered in the tumor tissues. Several LR pairs known to mediate immune cell crosstalk, such as CD86-CD28, CD40-CD40LG, ICAM1-ITGAL, and TNF-TNFRSF1A, exhibited increased signaling activity in the cancerous tissues. This suggests a heightened state of immune activation or compensatory signaling in response to tumor progression.

One of the most prominent changes was observed in CD86-CD28, a key co-stimulatory pathway essential for T-cell activation. There was a noticeable increase in the information flow in the cancer tissues compared to the normal tissues. This supports previous findings that *CD86* from macrophages helps boost immune reactions driven by T cells (14). Further, the TNF-TNFRSF1A signaling pathway, which is known for its dual role in inflammation and apoptosis (15), was also upregulated in the cancer microenvironment, indicating a potential feedback mechanism that may drive immune cell activation or exhaustion.

Interestingly, CD40-CD40LG interactions, which facilitate antigen-presenting cell activation and subsequent T-cell priming (16), showed a more pronounced engagement in the TAMs, further supporting their

immunomodulatory role in tumors. In contrast, adhesion-related signaling, such as ICAM1-ITGAL, was also significantly increased in the cancerous tissues, potentially contributing to enhanced immune cell recruitment and interaction stability.

Conversely, certain pathways, including transforming growth factor beta-transforming growth factor beta receptor (TGF β -TGF β R) and major histocompatibility complex class II (MHC-II) interactions, showed relatively lower information flow in the cancer tissues, which suggests that immune evasion strategies are employed by tumors to suppress antigen presentation and inhibit anti-tumor immunity.

Taken together, these results showed that the way in which macrophages and T cells communicate in tumors changes a great deal. The increased engagement of co-stimulatory and pro-inflammatory pathways suggests an attempt to mount an immune response against the tumor. However, the concurrent alterations in immunosuppressive signaling pathways indicate a complex interplay that may ultimately contribute to T-cell dysfunction or exhaustion, a hallmark of the immunosuppressive TME. Further investigations into the functional consequences of these interactions could provide valuable insights for therapeutic interventions targeting macrophage-T cell crosstalk in cancer immunotherapy.

Macrophage-T cell LR interactions in tumor and normal microenvironments

The LR interaction analysis revealed important changes in communication in the tumor tissues compared to the normal tissues (Figure 4A). Among the key LR pairs, SPP1-CD44, SPP1-(ITGA4 + ITGB1), NECTIN2-TIGIT, HLA-F-CD8B, HLA-E-CD8B, HLA-C-CD8B, HLA-B-CD8B, and HLA-A-CD8B exhibited increased interactions between macrophages and T cells in tumor tissues. In contrast, interactions such as SIGLEC1-SPN, junctional adhesion molecule 1 (JAM1)-(ITGAL + ITGB2), HLA-E-CD94:NKG2C, and HLA-E-KLRC2 were significantly reduced, reflecting the immunomodulatory role of macrophages in modulating T-cell activity within the TME.

In the tumor environment, SPP1-CD44 was significantly enriched; the *SPP1* (osteopontin) secreted by the TAMs was found to bind to *CD44* on the T cells. This interaction promotes T-cell adhesion and migration, while contributing to ECM remodeling and tumor progression. The enhanced SPP1-CD44 interaction aligns with the M2 polarization

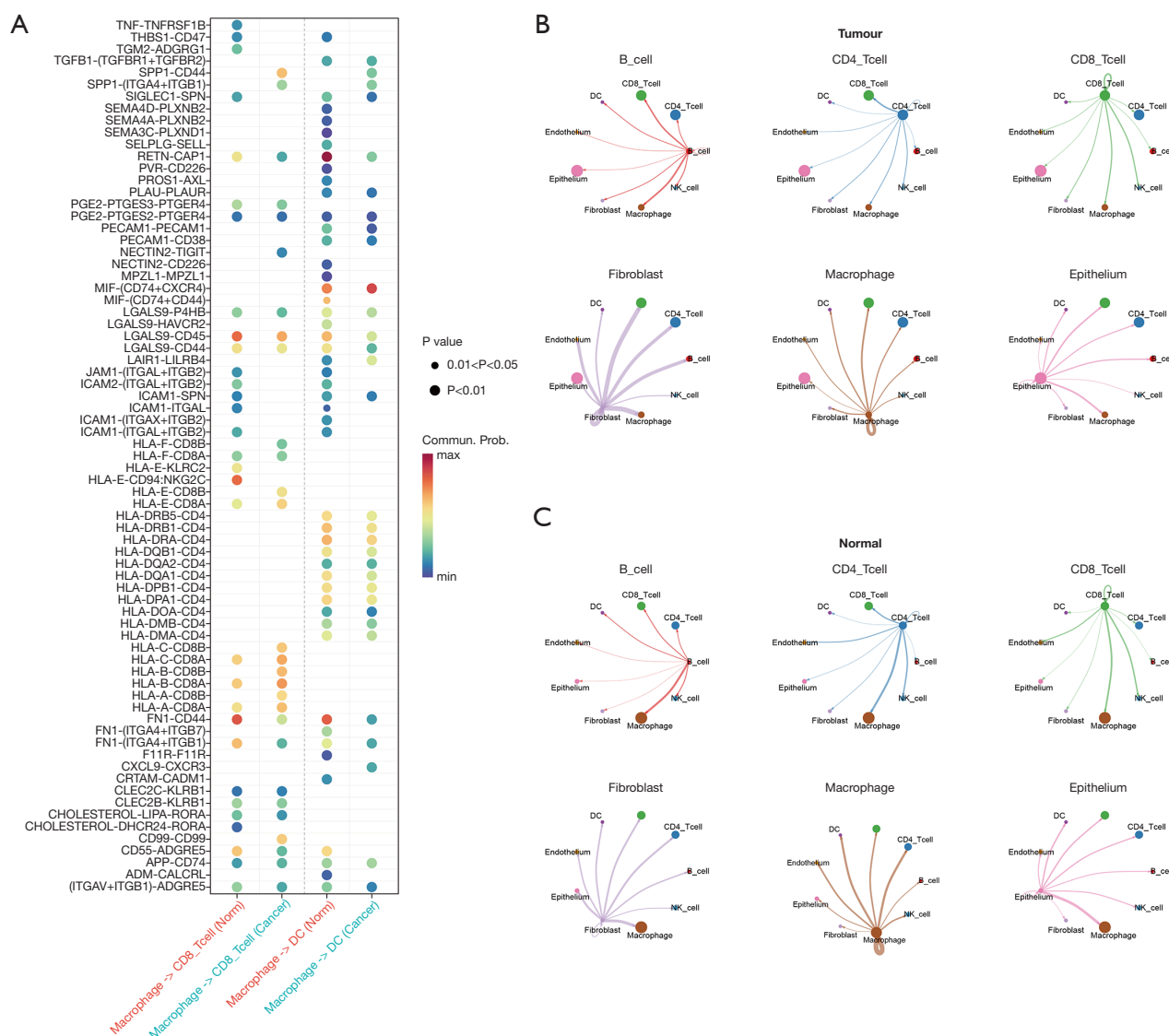


Figure 4 Macrophage-T cell receptor-ligand interactions in tumor and normal microenvironments. (A) Dot plot showing significant macrophage-T cell receptor-ligand interactions. The dot size represents the P value, and the color indicates communication probability. (B) Network of macrophage-T cell interactions in the tumor microenvironment. (C) Network of macrophage-T cell interactions in the normal microenvironment. DC, dendritic cell.

of macrophages, known to support immune suppression and tissue remodeling. Similarly, *NECTIN2*-*TIGIT* was strongly upregulated in the tumor tissues. *NECTIN2*, expressed by macrophages, interacts with *TIGIT* on T cells, reducing the activity of CD8⁺ T cells and boosting the immune-suppressing effects of Tregs. This interaction represents a central mechanism of immune escape in the areas around tumors, and thus could be a target for treatment.

In addition to macrophage-T cell interactions, broader changes in the cellular communication of tumors were found (Figure 4B,4C, and Figure S3B). The TAMs showed enhanced interactions not only with T cells but also with fibroblasts (*SPP1*-*ITGA4*/*ITGB1*) and epithelial cells (*SPP1*-*CD44*), suggesting their involvement in ECM remodeling and tumor invasion. The upregulation of *NECTIN2*-*TIGIT* further revealed an immunosuppressive shift. *NECTIN2* (*CD112*) is an immunoregulatory molecule

expressed in tumor and myeloid cells. It engages TIGIT, an inhibitory receptor on T cells, thereby reducing their proliferation and effector function (17). This increased association indicates that tumor-related macrophages might promote T-cell fatigue by promoting TIGIT signaling, which in turn weakens the ability of the body to fight tumors.

Elevated HLA-F-CD8B and HLA-E-CD8B interactions indicate altered antigen presentation and immune regulation in tumors. HLA-F and HLA-E are non-classical MHC class I molecules that engage CD8B, modulating CD8⁺ T-cell activation (18). HLA-E also interacts with CD94/NKG2 complexes, regulating cytotoxic lymphocyte function (19). The increased interaction involving HLA-E suggests that it plays a dual role in modulating CD8⁺ T-cell responses, such that it sustains antigen presentation while simultaneously contributing to immune evasion through inhibitory receptor engagement.

Conversely, key LR interactions were downregulated in tumors. The reduction of SIGLEC1-SPN suggests a decreased macrophage-mediated immunosuppressive interaction, as SIGLEC1 (CD169), an inhibitory receptor expressed on M2-like macrophages, binds to SPN (CD43) in T cells to suppress activation. The downregulation of this interaction may indicate an altered immunosuppressive landscape, possibly influenced by therapeutic interventions or dynamic immune adaptation in tumors. Similarly, JAM1-(ITGAL + ITGB2) downregulation suggests impaired leukocyte adhesion and migration. JAM1 facilitates T-cell transendothelial migration by interacting with LFA-1 (ITGAL + ITGB2) in T cells. A decrease in this interaction could limit T-cell infiltration into tumors, further restricting anti-tumor immunity.

Additionally, the weaker links between HLA-E and CD94:NKG2C, as well as HLA-E and KLRC2, indicate that there is less activation of certain immune cells that attack and destroy other cells. The CD94/NKG2C complex is a protein found in NK cells and CD8⁺ T cells. It activates these cells by binding to HLA-E, which boosts their ability to kill target cells, while NKG2A, an inhibitory counterpart, inhibits immune activation. The observed reduction in HLA-E engagement with activating receptors suggests a shift toward immune suppression, favoring tumor immune evasion.

Taken together, these findings illustrate a profound remodeling of macrophage-T cell interactions in the area around the tumor. The enrichment of immunosuppressive interactions (SPP1-CD44 and NECTIN2-TIGIT), as

well as the loss of key adhesion and activating signals (SIGLEC1-SPN, JAM1-ITGAL + ITGB2, and HLA-E-NKG2C), suggests that macrophages actively shape T-cell responses by promoting exhaustion, restricting infiltration, and modulating antigen presentation. These changes show how important communication between macrophages and T cells is for regulating the immune system in tumors. They also suggest possible new treatment options for adjusting immune reactions in cancer.

Spatial distribution and abundance of macrophages and immune cells across tissue types

The spatial transcriptomics analysis revealed distinct distribution patterns and expression levels of macrophages, DCs, CD4⁺ T cells, CD8⁺ T cells, and NK cells in the normal, peritumoral, and tumoral tissues (*Figure 5A*). The macrophages showed high spatial overlap with other immune cells in all tissue types, but their expression levels progressively increased from the normal to peritumoral and tumor tissues, showing their key role in the TME. Conversely, the DCs, T cells, and NK cells showed a steady decline in terms of their amounts and numbers as the tissues changed from normal to tumor tissue, suggesting that macrophages actively reshape the immune microenvironment. In the normal tissues, the macrophages were evenly distributed and displayed relatively low abundance, coexisting with other immune cells in a balanced immune surveillance environment. However, in the peritumoral tissues, macrophage density increased significantly, particularly at the tumor border, indicating the role of macrophages in early immune modulation. In the tumor tissues, the macrophages were densely concentrated, especially at the tumor core, where they formed a dominant cellular network indicative of their transformation into TAMs with immunosuppressive phenotypes.

The DCs were present across all tissue types but showed progressively reduced expression levels from the normal to tumor tissues. In the normal tissues, the DCs overlapped spatially with the macrophages, and likely supported immune surveillance, but in the tumor tissues, their diminished expression indicated a loss of antigen-presenting capacity. The CD4⁺ T cells showed significant spatial overlap with macrophages in all tissue types, but their abundance decreased markedly in the tumor tissues, particularly in the macrophage-dense regions, suggesting that macrophages may suppress CD4⁺ T-cell helper functions. Similarly, the CD8⁺ T cells, which were widely

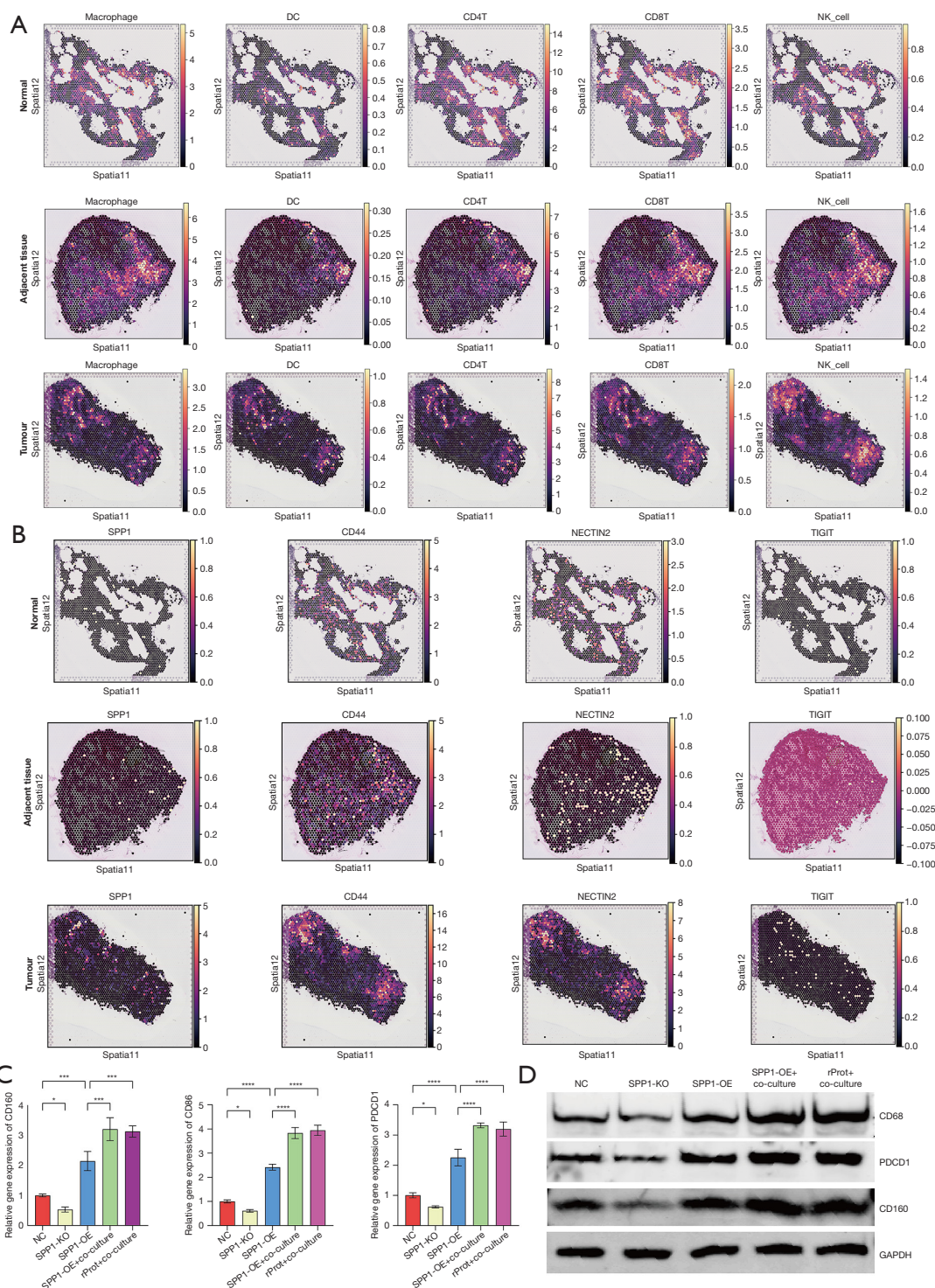


Figure 5 SPP1-mediated epithelial cell regulation enhances macrophage-T cell immunosuppression in the tumor microenvironment. (A) Spatial transcriptomics analysis showing the distribution and abundance of macrophages and immune-related markers across normal, adjacent, and tumor tissues. The color intensity represents gene expression levels in tissue sections. (B) SPP1-CD44 and NECTIN2-TIGIT interactions in the tumor microenvironment. (C) Quantitative polymerase chain reaction analysis of SPP1 and immune-related genes. (D) Western blot analysis of SPP1 and downstream signaling molecules. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$. DC, dendritic cell; NC, negative control; OE, overexpression.

distributed in the normal tissues, became progressively restricted in the peritumoral and tumor regions. In the tumor tissues, CD8⁺ T cells showed limited abundance, especially in the macrophage-enriched regions, indicating that the macrophages might inhibit cytotoxic T-cell infiltration and activation. The NK cells were sparsely distributed across all tissue types, and only very small amounts were observed in the tumor cells. The NK cells presented in modest amounts, and shared some locations with the macrophages in the normal tissues. The near absence of NK cells in tumor tissues suggests that macrophages may contribute to their exclusion or functional suppression.

These findings show that macrophages dominate the immune microenvironment by increasing their abundance and altering the functional states of other immune cells. The progressive reduction in DCs, T cells, and NK cells across tissue types highlights macrophage-mediated immune suppression, potentially through cytokine signaling (e.g., IL-10 and TGF- β) and immune checkpoint interactions. In normal tissues, macrophages coexist with other immune cells to maintain immune surveillance, while in peritumoral tissues, macrophages appear to orchestrate early immune modulation by interacting with T cells and DCs. In tumor tissues, macrophages establish an immunosuppressive microenvironment, suppressing cytotoxic T-cell activity and excluding NK cells. These findings highlight the critical role of macrophages in remodeling the immune landscape, and suggest that targeting macrophage-T cell and macrophage-NK cell interactions could reinstate immune surveillance and enhance anti-tumor immunity.

Epithelial cells are key players in the communication between macrophages and T cells in tumors

This study examined how macrophages, epithelial cells, and T cells interact in tumors. Specifically, it examined the expression and placement of important LR pairs (Figure 5B and Figure S4), including SPP1-CD44, NECTIN2-TIGIT, HLA-F-CD8B, HLA-E-CD8B, and HLA-E-KLRC2 in the normal, adjacent, and cancerous tissues. The spatial transcriptomic analysis showed that the expression of these interaction pairs increased from the normal tissue to the adjacent tissue, with the highest expression levels observed in the tumor tissue, indicating a remodeling of immune-epithelial interactions during tumor progression. SPP1 is primarily secreted by TAMs. It shows strong colocalization with CD44-expressing epithelial and immune cells in

tumor regions. This suggests that SPP1-CD44 signaling contributes to an immune-modulating niche that promotes tumor progression (20). CD44, a popular marker for cancer stem-like cells was found in high levels in epithelial cells and was also noticeably present in CD8⁺ T cells. This means that the SPP1-CD44 pathway helps tumor cells live longer and also affects the way in which immune cells function (21). The elevated expression of CD44 in tumors is aligned with its involvement in epithelial-mesenchymal transition, ECM remodeling, and resistance to immune surveillance.

The NECTIN2-TIGIT axis was also highly enriched in tumors; *NECTIN2* was predominantly expressed by epithelial cells and myeloid-lineage cells, while *TIGIT* was upregulated in CD8⁺ T cells and NK cells. The strong colocalization of NECTIN2-expressing tumor cells with TIGIT⁺ immune cells suggests that tumor epithelial cells suppress T- and NK-cell cytotoxicity via TIGIT-mediated inhibition. This leads to an exhausted T-cell phenotype with reduced effector function. The increased expression of *TIGIT* in tumors, coupled with its colocalization with CD8B, suggests a shift toward immune suppression, allowing tumors to evade T cell-mediated killing. Further, the expression of *HLA-F* and *HLA-E*, two non-classical MHC-Ib molecules, was increased in the tumor epithelial cells, while *CD8B* expression was relatively lower in the same cells, indicating the potential depletion or suppression of cytotoxic T cells. *HLA-F* was found to be colocalized with CD8B⁺ T cells in the adjacent and tumor tissues, suggesting that epithelial cells may modulate T-cell activity through *HLA-F*-mediated immune regulation. Similarly, *HLA-E* exhibited a strong colocalization pattern with *KLRC2* (encoding *NKG2A*) in the tumors, indicating an enrichment of NK cells expressing inhibitory receptors. The increased expression of *HLA-E* in epithelial cells, along with the increased expression of *NKG2A* in NK cells, reinforces the hypothesis that tumor cells exploit *HLA-E*/*NKG2A* interactions to suppress NK cell cytotoxicity and evade immune surveillance.

Collectively, these findings show that epithelial cells play an important role in helping macrophages and T cells communicate with each other in the tumor environment. Tumor epithelial cells actively engage with macrophages and T cells via multiple LR interactions, fostering an immunosuppressive microenvironment that facilitates tumor progression. The upregulation of SPP1-CD44 promotes an immune-modulating niche that supports tumor growth and immune evasion, while NECTIN2-TIGIT and *HLA-E*/*NKG2A* interactions cause CD8⁺ T cells and NK

cells to become less effective, respectively. These results indicate that focusing on the immune suppression caused by epithelial cells could be a potential way to boost the ability of the body to fight tumors.

Macrophage-derived SPP1 regulates epithelial and T-cell interactions to drive immune suppression and tumor progression

To validate this regulatory mechanism, macrophages (derived from THP-1 cells) were genetically manipulated to either KO or overexpress the *SPP1* gene, and subsequently co-cultured with epithelial cells and T cells. The RT-qPCR analysis (Figure 5C) revealed significant changes in immune suppression markers, such as *PDCD1* and *CD160*, under these conditions. Specifically, the OE of *SPP1* in macrophages, particularly in co-cultures with epithelial cells and T cells, led to a marked increase in *PDCD1* and *CD160* expression levels ($P < 0.001$). Conversely, the KO of *SPP1* in macrophages significantly reduced the expression of these markers, suggesting that macrophage-derived *SPP1* modulates epithelial cell and T-cell interactions to enhance immunosuppression.

The Western blot analysis (Figure 5D) further corroborated these findings. The study examined protein levels across the following five experimental groups: NC (negative control), *SPP1-KO*, *SPP1-OE*, *SPP1-OE* + co-culture, and recombinant protein + co-culture. Notably, the highest amounts of *CD68*, *PDCD1*, and *CD160* were observed in the recombinant protein and co-culture group, followed by the *SPP1-OE* and co-culture group. This pattern indicates that macrophage-derived *SPP1* plays a critical role in regulating the immunosuppressive microenvironment by influencing T-cell activity and epithelial cell behavior. The findings suggest that *SPP1* enhances macrophage-mediated immune suppression, further reinforcing the role of macrophages in promoting tumor survival and progression.

These results show that macrophages play a crucial part in connecting epithelial cells and T cells, driving immune suppression, and influencing tumor progression through *SPP1*-mediated regulatory mechanisms.

Discussion

This study integrated scRNA-seq and spatial transcriptomics data to uncover key cellular interactions and immunosuppressive mechanisms in the areas surrounding

a tumor and thus establish a high-resolution immune atlas of NSCLC. By studying immune and non-immune cells in terms of their gene activity and location, we identified significant alterations in cell composition, LR interactions, and immune exclusion patterns, providing novel insights into the progression of NSCLC. Previous studies have highlighted that certain cells, such as TAMs, Tregs, and fibroblasts, help tumors escape the immune system by changing the structure around them and promoting blood vessel growth, and suppressing cytotoxic T-cell responses (12,22,23). In line with these findings, our study confirmed that in the NSCLC microenvironment, antigen-presenting molecules (e.g., *HLA-DRA* and *CD74*) and immunosuppressive cytokines (e.g., *IL-10* and *TGF- β*) are highly expressed in TAMs, while metabolic regulators (e.g., *APOE* and *SPP1*) show signs of exhaustion in tumor-infiltrating T cells, indicating these T cells have higher levels of immunity checkpoints (e.g., *TIGIT*, *CTLA4*, and *PDCD1*) and lower levels of markers that help them fight cancer (e.g., *GZMB* and *PRF1*). These results further support the notion that NSCLC actively reprograms immune responses to evade immune surveillance.

Beyond these expected observations, this study presented several novel findings that extend our understanding of NSCLC immune regulation. One of the most significant discoveries was the identification of *SPP1* as a central regulator of macrophage-T cell interactions. The role of *SPP1* has not been fully explored in NSCLC (24). Previous studies have shown that *SPP1* is involved in changing the structure of the ECM and in the spread of cancer; however, its function in immune suppression was not fully understood (25). Our spatial transcriptomic data reveal that *SPP1* is primarily secreted by TAMs rather than epithelial cells, a finding consistent with previous reports that *SPP1*⁺ macrophages are enriched in NSCLC and promote immune evasion. Notably, we observed a strong colocalization of *SPP1*-expressing macrophages with *CD44*⁺ T cells in tumor regions, suggesting that the *SPP1*-*CD44* axis is a key mechanism underlying T-cell dysfunction in NSCLC (26,27). This builds on previous findings that *SPP1* enhances M2 macrophage polarization and supports a pro-tumoral microenvironment (28). Our findings extend this knowledge by providing spatial evidence that *SPP1*-*CD44* signaling may actively suppress T-cell activity, supporting the rationale for targeting the *SPP1*-*CD44* axis to improve immunotherapy responses (29).

Another major finding of this study was that the *NECTIN2*-*TIGIT* interaction between macrophages

and T cells is a dominant immunosuppressive pathway in NSCLC, which was validated at the spatial level. Previous studies have identified *TIGIT* as a key immune checkpoint in exhausted T cells (30); however, our findings provide new evidence that TAMs actively contribute to T-cell exhaustion by upregulating *NECTIN2* expression, thereby suppressing cytotoxic T-cell function. The strong colocalization of *NECTIN2*-expressing macrophages with *TIGIT*⁺ CD8⁺ T cells suggests that this interaction plays a crucial role in immune evasion rather than merely reflecting a passive state of T-cell exhaustion. The *TIGIT* blockade is currently under investigation in clinical trials (31,32). Our findings provide a strong rationale for combining anti-*TIGIT* therapy with macrophage-targeting strategies to enhance immune responses in NSCLC.

In addition to macrophage-T cell interactions, our analysis revealed HLA-E-CD8B interactions as a previously underappreciated immune evasion mechanism in NSCLC. While HLA-E is known to regulate NK- and T-cell activity (33), our findings indicate that HLA-E-CD8B⁺ T-cell interactions are significantly enriched in tumors, potentially serving as a mechanism for modulating antigen presentation and promoting immune tolerance. This observation aligns with emerging evidence that HLA-E engagement with inhibitory receptors (e.g., NKG2A) may contribute to tumor immune evasion (34). The increased interaction between HLA-E and CD8B⁺ T cells in NSCLC suggests that tumor cells may exploit this pathway to impair CD8⁺ T cell-mediated cytotoxicity, offering a potential target for future immunotherapeutic interventions.

A key advantage of this study is the integration of spatial transcriptomics, which enabled the direct visualization of immune exclusion mechanisms in NSCLC tumors. Unlike previous scRNA-seq studies that have relied solely on dissociated single cells, our spatial data revealed that macrophage-T cell interactions are highly localized to tumor cores, while cytotoxic T cells are predominantly restricted to normal tissues. This finding supports the concept of immune-excluded tumors, where macrophages act as both physical and biochemical barriers to T-cell infiltration. Previous research suggests that fibrotic barriers and dysfunctional vasculature contribute to immune exclusion in NSCLC (35,36). Our study provides further evidence that macrophages may directly limit T cells from reaching tumor areas. These results are very important for chemotherapy, and suggest that simply reinvigorating T cells with checkpoint inhibitors may not be sufficient unless macrophage-mediated immune exclusion is also targeted.

To more closely examine how SPP1 helps silence the immune system, we conducted studies in which the SPP1 levels in macrophages made from THP-1 cells were changed. These macrophages were grown together with epithelial cells and T cells. Our qPCR and Western blot analyses confirmed that the OE of *SPP1* in macrophages significantly increased *PDCD1* and *CD160* expression, while its KO reduced these immune suppression markers. The results showed that a substance called *SPP1*, which is produced by macrophages, is important for creating a tumor environment that weakens the immune response. This finding highlights its promise as a target for new treatments. In addition, accumulating evidence indicates that SPP1 may exert its immunosuppressive effects through several downstream signaling pathways. For instance, activation of the PI3K/AKT/mTOR axis can promote T-cell dysfunction and tumor cell survival; NF-κB signaling may drive the production of immunosuppressive cytokines and sustain chronic T-cell exhaustion; and integrin-mediated signaling downstream of SPP1-CD44 interactions can remodel the ECM and facilitate immune evasion. Although our study did not directly dissect these mechanisms, future work should systematically evaluate these pathways to clarify how SPP1 shapes the immunosuppressive microenvironment in NSCLC. The implications of these findings for NSCLC immunotherapy are substantial. The identification of SPP1-CD44, *NECTIN2*-*TIGIT*, and HLA-E-CD8B as key immunosuppressive pathways suggests that targeting these interactions may restore T-cell infiltration, enhance cytotoxic function, and overcome macrophage-mediated immune suppression. Given the increasing interest in combination immunotherapies, our findings strongly support the use of SPP1 blockers along with immune checkpoint treatments like anti-PD-1 or anti-*TIGIT* therapy to enhance patient outcomes.

In addition, it is important to consider how these newly identified immunosuppressive axes interact with established immune checkpoints. Our findings suggest that the SPP1-CD44 and *NECTIN2*-*TIGIT* pathways may act synergistically with classical checkpoints such as PD-1/PD-L1 and CTLA-4, thereby reinforcing T-cell dysfunction, while the HLA-E-CD8B interaction appears to function independently by directly suppressing cytotoxic T-cell activity. These insights underscore the rationale for rationally designed combination strategies that target both established checkpoints and novel immunosuppressive pathways to achieve more effective immunotherapy in NSCLC.

Despite these advances, our study had some limitations. Although we provide strong evidence for key immunosuppressive interactions in NSCLC, additional *in vivo* validation is needed to confirm whether disrupting these pathways effectively restores anti-tumor immunity. Moreover, while our spatial transcriptomic approach enabled the high-resolution mapping of immune interactions, future studies should integrate multiplexed imaging or spatial proteomics to further validate these findings at the protein level. In addition, experimental confirmation of the scRNA-seq clustering results using flow cytometry or immunohistochemistry will be important to further strengthen the robustness of our cell-type annotation. Beyond the validation of SPP1, other predicted immunosuppressive axes such as NECTIN2-TIGIT and HLA-E-CD8B represent important directions for future research, and our integrative single-cell and spatial analysis provides a solid computational biology foundation to guide these future efforts.

Conclusions

In conclusion, this study constructed a comprehensive immune atlas of NSCLC, revealing novel macrophage-T cell interactions that drive immune suppression in tumors. By identifying SPP1-CD44 as a key regulatory axis, and uncovering macrophage-driven NECTIN2-TIGIT and HLA-E-CD8B interactions, we provided novel insights into the mechanisms of NSCLC immune evasion. These findings highlight macrophage-T cell crosstalk as a promising therapeutic target and suggest that disrupting immunosuppressive interactions may improve the effectiveness of immunotherapy for NSCLC patients. Our study lays the groundwork for precision medicine strategies aimed at reprogramming tumors to improve patient outcomes.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki and its subsequent amendments.

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