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Single-cell RNA sequencing reveals immune microenvironment niche transitions during the invasive and metastatic processes of ground-glass nodules and part-solid nodules in lung adenocarcinoma

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Abstract

Background Radiographically, ground-glass nodules (GGN) and part-solid nodules (PSN) in lung adenocarcinoma (LUAD) have significant heterogeneity in their clinical manifestations, biological characteristics, and prognosis. This study aimed to explore the heterogeneity of LUAD in different radiological phenotypes and associated factors influencing tumor evolution.

Methods We performed single-cell RNA sequencing (scRNA-seq) on tumor tissues from eight and seven cases of GGN- and PSN-LUAD, respectively, at different disease stages, including minimally invasive adenocarcinoma (MIA), invasive adenocarcinoma (IAC), and metastatic lung cancer (MLC). Additionally, we analyzed adjacent normal tissues from four cases. Immunohistochemistry, multiplex immunofluorescence, and external scRNA-seq data were employed to confirm the expression of signature genes as well as the distribution patterns of CXCL9 + TAMs and TREM2 + TAMs. A LUAD mouse model was generated using gene editing, organoid culture, and orthotopic transplantation techniques, and comprehensive analyses such as histopathology, RNA sequencing, and Western blotting were performed to validate key pathways.

Results Diverse cellular compositions were observed in the tumor microenvironment (TME) during GGN- and PSN-LUAD invasion and metastasis. Notably, CXCL9 + and TREM2 + tumor-associated macrophages (TAMs) exhibited the most significant enrichment changes. It was found that GGN-LUAD exhibited a stronger immune response than PSN-LUAD, with increased interaction between CXCL9 + TAMs and CD8 + tissue-resident memory T cells during invasion stage (MIA-IAC). Conversely, greater interactions between TREM2 + TAMs and tumor cells were observed in PSN-LUAD during the MLC stage. Additionally, TREM2 + TAMs were found to differentiate into TREM2 + /SPP1 + and

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TREM2 +/SPP1– TAMs at different stages, which promotes tumor progression. This study also emphasizes that during the transdifferentiation process of GGN– and PSN–LUAD, IFN- γ activates the STAT1 signaling pathway to regulate the activation of CXCL9 + TAMs, and further recruiting CD8 + Trm cells and activating T cells through MHC class I antigen presentation. The role of the IFN- γ /STAT1 pathway in the occurrence and development of LUAD was further validated by animal experiments.

Conclusions Our findings offer a potential therapeutic strategy to maintain a dynamic balance within the TME and improve the immunotherapy efficacy by modulating the relative proportions and functional states of CXCL9 + TAMs and TREM2 + TAMs.

Keywords Lung adenocarcinoma, Pulmonary nodule, Tumor microenvironment, Single-cell RNA sequencing, CXCL9, TREM2, SPP1, Tumor-associated macrophage

Background

With the widespread use of high-resolution low-dose computed tomography, an increasing number of lung adenocarcinoma (LUAD) cases are being detected in the form of pulmonary nodules, among which ground-glass nodules (GGN) and part-solid nodules (PSN) are the two most frequently detected types, accounting for 31.7–69.7% of all pulmonary nodules [1, 2]. Notably, early-stage LUAD with GGN features exhibit a nearly 100% 5-year disease-free survival rate post-surgery. In contrast, PSN with more solid components show a higher risk of metastasis and disease progression [3, 4]. This suggests a potential strong correlation between different radiological features and the malignant phenotype and disease progression of LUAD.

The tumor microenvironment (TME) of LUAD is composed of cancer cells, infiltrating immune cells, stromal cells, and other cellular types, as well as non-cellular tissue components, which interact with each other and collectively determine the initiation and progression of the tumor [5, 6]. Single-cell RNA sequencing (scRNA-seq) technologies enable the evaluation of thousands of cells within a single sample, thereby revealing the heterogeneity of tumor cells and the subtle nuances of the complex TME [7]. The development of LUAD is often considered to progress from adenocarcinoma in situ (AIS), through the minimally invasive adenocarcinoma (MIA) stage, to invasive adenocarcinoma (IAC), and ultimately to distant metastatic lung cancer (MLC) [8, 9]. Currently, studies have utilized scRNA-seq technologies to reveal the evolutionary trajectories from pre-invasive to invasive LUAD. For example, Zhu et al. studied the cellular ecologies and spatial niches involved in the dynamic and sequential processes of LUAD from AIS to MIA and subsequent IAC [10]. Wu et al. mapped cell type-specific scRNA-seq profiles of cancer cells and their tumor microenvironment in advanced non-small cell lung cancer (NSCLC) [11]. Furthermore, Li et al. revealed the multi-cellular ecosystems within different radiological components of PSN. However, these studies are not comprehensive and

do not fully capture the progression of LUAD from its early stages, nor do they identify the characteristics of the same type of pulmonary nodule across different pathological periods [12]. To our knowledge, there is currently no comprehensive study evaluating the effects of GGN and PSN features on the molecular and immunological characteristics of MIA, IAC, and MLC-LUAD.

To delineate the evolutionary trajectories of GGN- and PSN-LUAD, we generated single-cell transcriptomic atlases of tumor tissues and normal lung tissues across various stages of disease progression (MIA/IAC/MLC) for both GGN and PSN-LUAD. These atlases capture the heterogeneity of the TME cell populations during the invasion and metastasis of LUAD with distinct radiological characteristics. Leveraging publicly available patient sequencing data and multiplex immunofluorescence technologies, we further determined the distribution patterns of TME cell. Within this extensive heterogeneity, we identified key tumor-associated macrophage (TAM) subsets-TREM2 + TAMs and CXCL9 + TAMs-that drive either tumor-suppressing or promoting phenotypes, and elucidated the underlying mechanisms regulating their differentiation using organoids and gene-editing techniques. In summary, our analysis provides an in-depth characterization of the molecular features of the evolutionary trajectories of GGN- and PSN-LUAD, with significant clinical implications.

Methods

Patients and specimens

This study included nineteen LUAD patients with SSN who underwent curative resection surgery at the Department of Thoracic Surgery, Hospital of Chengdu University of Traditional Chinese Medicine, and Sichuan Cancer Hospital between June 2022 and May 2023, as confirmed by pathology. The study adhered to the principles of the Declaration of Helsinki and received approval from the Institutional Review Boards (IRBs) of Hospital of Chengdu University of Traditional Chinese Medicine (IRB No. 2022KL-051) and Sichuan Cancer Hospital (IRB

No. SCCHEC-02–2022-109). Patient demographics and clinicopathological characteristics are presented in Supplementary Table S1.

Patients were categorized into GGN– or PSN–LUAD groups with various subtypes: three, three, and two with GGN–MIA, GGN–IAC, GGN–MLC; and three, three, and one with PSN–MIA, PSN–IAC, PSN–MLA. Normal non-neoplastic tissues from four patients (two each) were collected over 2 cm from tumor edges, evaluated cryopathologically, and classified per the 2015 WHO system.

Mice

Mice were housed in a specific pathogen-free animal facility at Hospital of Chengdu University of Traditional Chinese Medicine, where they were provided with autoclaved food, bedding, and water. All mouse experiments were performed in accordance with guidelines approved by the Institutional Animal Care and Use Committees of Hospital of Chengdu University of Traditional Chinese Medicine, with protocols approved by the Animal Care and Use Committee of TCM Regulating Metabolic Diseases Key Laboratory of Sichuan Province, Hospital of Chengdu University of Traditional Chinese Medicine. C57BL/6 and CAG-Cas9-EGFP mice (male, 6–8 weeks old, approximately 20 g in weight) were used. Mice were purchased from GemPharmatech (RRID: IMSR_JAX:026179). Tumor volumes did not exceed the maximal permitted volume of 1,000 mm³.

Preparation of single-cell suspensions

After surgery, tissue samples were split: one part was fixed for immunofluorescence, and the other was placed in ice-cold H1640 (Gibco) for single-cell suspensions. Tissues were minced, digested with the GentleMACS Dissociator, then washed, filtered, and centrifuged before being resuspended in a solution with 0.2% foetal bovine serum. Cells were manually counted, and processed using the Chromium Controller (10×Genomics). Single cells were processed using the Chromium Controller according to the manufacturer's protocol.

Library preparation and scRNA-seq

Prepared cell suspensions and reaction mixtures (Master Mix + Sample), 10×barcode Gel Beads (VDJ 5' Gel Beads v1.1), and partitioning oil were added to different wells of the Chromium Next GEM Chip. Through the microfluidic "single cross" system, GEMs were formed. Then, Gel beads were dissolved and cells were lysed within each GEM. The released oligonucleotides mixed with the lysed cells and Master Mix (containing RT reagents and RT primers) to undergo reverse transcription, resulting in full-length complementary DNA (cDNA) sequences with 10×barcodes. After reverse transcription, the Recovery

Agent was used to disrupt the oil-in-water structure, and recover and purify the cDNA. Subsequently, the cDNA was amplified under the action of DNA polymerase. The amplified cDNA is then fragmented and selected for appropriate fragments. After end repair and A-tailing, Read2 sequencing primers were ligated, and Polymerase Chain Reaction (PCR) was performed to construct a cDNA library containing P5 and P7 adapters.

scRNA-seq data pre-processing

Cell Ranger software (<https://support.10xgenomics.com/single-cell-vdj>) was utilized to align and annotate reads using human genome assembly GRCh38p13, and gene annotation GENCODE34 was used to generate count matrices. Once aligned, the barcodes associated with read-cell identifiers and unique molecular identifiers (UMI) were processed in R (version 3.6.0) using the Seurat R package (version 4.0.0) [13]. The cells with mitochondrial UMI counts > 25%, or < 500 genes were identified as low-quality cells and removed. DoubletFinder was used to remove doublets. After removing low-quality cells, the gene expression matrix was normalized using the normalization function, and the top 2000 features with high cell-to-cell variability were calculated using the Seurat normalization function.

Dimension reduction and identification of major cell clusters

Dimensionality reduction was performed by principal component analysis. Subsequently, we applied the Harmony algorithm, a batch effect correction method, to refine the integration. Utilizing the Harmony results as a foundation, clustering analysis was conducted using standard Seurat package procedures. We employed the Clustree package to construct a dendrogram by specifying distinct levels of clustering, facilitating a clear visualization of the interrelationships among various clustering tiers. Ultimately, for different cellular phenotypes, we selected appropriate resolution parameters to enable annotations that are biologically significant: T cells (0.8), B cells (0.05), endothelial cells (0.05), epithelial cells (0.8), and myeloid cells (0.5). After non-linear dimensional reduction and projection of all cells into a two-dimensional space through uniform manifold approximation and projection (UMAP), cells with common features were clustered together in the UMAP map. The differentially expressed genes (DEGs) of each cluster were identified by the "FindAllMarkers" function in Seurat, and the clusters were annotated according to the expression of classic markers (parameters: only.pos = FALSE, min.pct = 0.1, thresh.use = 0.1). Clusters were classified and annotated based on the expression of canonical markers in the particular cell types. Cell-type markers were

adapted from Hu et al. [14]. Doublets and low-quality cell clusters were excluded from further analysis.

Differentially expressed pathway analysis between different clinical groups

Gene set variation analysis (GSVA) was used to assess differential pathways, and the activity scores of special cells were calculated by GSVA (v1.40.1) (false discovery rate (FDR) < 0.05) [15]. Significantly enriched pathways were defined at an adjusted p (p_{adj}) value < 0.05. The Gene Ontology (GO) gene sets were subjected to an enrichment analysis using the R package clusterProfiler version 3.16.1. To account for multiple comparisons, the p -values were adjusted using the Benjamini–Hochberg procedure. Ontologies were considered significant if the q -value was < 0.05.

Identification of cancer cells

To identify cancer cells, we used inferCNV (version 1.2.1) to search copy number variations (CNVs) in each subcluster of epithelial cell clusters from the scRNA-seq data. The raw count data was extracted from the Seurat object according to the recommendations in the "Use 10×Data" section. The epithelial cells in the normal control (NC) sample were used as reference cells, and density curves were plotted for the estimated CNV values, with 928.68 as the baseline value for distinguishing tumors from normal cells. We created a gene sorting file from the assembly of human CRCh38, containing the chromosome start and end positions of each gene. For inferCNV analysis, we used 0.1 as the cutoff value for the minimum average read count of each gene in the reference cell and denoised the output to predict CNV levels. In addition, CopyKAT was used to calculate the genomic copy number distribution of individual cells by combining Bayesian methods with hierarchical clustering and to define subclonal structures. CopyKAT can automatically identify diploid cells as normal. For each non-diploid cell, Markov chain Monte Carlo (MCMC) is used to identify the breakpoints of CNVs and to determine segments. Normal and tumor cells can be distinguished based on their different gene expression profiles. Ultimately, the results from inferCNV and CopyKAT were combined to define the tumor cell populations within the epithelial cells.

Immunohistochemistry and immunofluorescence staining

All dissected tissues were fixed in 4% PFA and embedded in paraffin. The tissues were cut into 4- μ m sections and stained with hematoxylin and eosin. Ki67 (Abcam, ab15580) markers were used for immunohistochemical analysis. Immunofluorescent staining was performed using the antibodies CD68 (ImmunoWay, YM3050), TREM2 (Proteintech, 22,952-1-AP), and CXCL9

(ImmunoWay, YT6008). Images were captured using a Nikon ECLIPSE C1 camera.

Pseudotime trajectory analysis

DEGs were identified by Seurat. The R package Monocle (version 2.4) was used with default settings to plot the pseudotime trajectory. Pseudotime ordering was performed using the function "reduce dimension" with max components set at 2 and the reduction method set as DDRTree. Next, the significantly affected genes were obtained from the top 50 markers among the clusters using the function differentialGeneTest (fullModelFormulaStr = ~Pseudotime) and were plotted with the function plot pseudotime heatmap.

Transcription factor analysis

Based on the scRNA-seq results, pySCENIC analysis was run using the motifs database for RcisTarget and GRNboost (pySCENIC version 0.1.1.2) with the default parameters 50. We regarded each regulatory network as a regulon. Through the analysis of the regulon activity in each cell type, we determined the differences in the regulatory activity of transcription factors among different cell types.

Cell–cell interaction score calculation using CellPhoneDB

We investigated cell–cell interactions across all cell types with CellPhoneDB (version 3.1.0) (<https://www.cellphonedb.org/>). To identify the most relevant interactions between cell types, CellPhoneDB identified cell type-specific interactions between ligands and receptors. Only receptors and ligands expressed in > 10% of the cells in the specific cluster were considered significant. CellPhoneDB then performed pairwise comparisons between all cell types. The ligand–receptor pairs with P value < 0.05 were retained. To assess the cell–cell interactions, we calculated the total interaction score of each cell pair: summation of the scores of all ligand–receptor pairs within a cell pair. The total score reflects the abundance and expression of ligand–receptor pairs within each cell pair, regardless of whether the interaction involves positive or negative regulatory ligands/receptors for antitumor immunity.

CellChat analysis

Cell–cell communication was analyzed using the CellChat pipeline with version 1.1.3. A new CellChat object was generated from the consolidated Seurat object. The CellChatDB's dataset of paracrine/autocrine signaling interactions was utilized as the reference database. Subsequently, the communication probabilities were calculated with a 20% truncated mean (using the computeCommunProb function with type set to "truncatedMean" and trim

set to 0.2). Cell–cell communication was then inferred, and the network was aggregated with default parameters. The number of interactions was visualized to depict the aggregated cell–cell communication network and the signals sent from each cell cluster.

Cell culture

HEK 293 T cells (CRL-1573) from ATCC were obtained and cultured in DMEM supplemented with 10% (vol/vol) fetal bovine serum and penicillin (100 U ml⁻¹)/streptomycin (0.1 mg ml⁻¹), and were incubated at 37 °C, 5% CO₂ cell incubator.

Lung organoid culture

Mouse lungs were removed, cut into 3mm³ pieces and incubated with digestion buffer containing 1.0 mg ml⁻¹ collagenase I (Gibco, 17,100–017) and 0.5 mg ml⁻¹ collagenase IV (Gibco, 17,104–019) in DMEM/F12 (Gibco, C11330500BT) at 37 °C for 45 min. After dissociation, the samples were filtered through 100-μm filters to obtain a cell suspension, then centrifuged and washed once with DMEM/F12. After solidification, 150 μl of organoid expansion medium (ExM) was added to each well. The ExM and growth factors were generously provided by Prof. Chen [16]. Organoids were passaged via mechanical dissociation in TrypLE (Gibco, 12,605–028) every 5–7 days.

Genomic DNA extraction, PCR amplification

Five hundred microliters DNA lysis buffer (homemade, 10 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.5% SDS 0.4 mg/ml proteinase K) were used to lyse the cells at 55 °C for at least 2 h. The supernatant was combined with 500 μl isopropanol, mixed, and centrifuged at 13,000 rpm for 10 min. The supernatant was removed, and the sample was washed once with 1 mL of 75% ethanol. The sample was volatilized at 55 °C for approximately 5 min, and the DNA pellet was subsequently dissolved in double-distilled water. One hundred nanograms DNA was used for each genotyping PCR reaction. PCR reactions were performed using a ProFlex™ PCR system (Applied Biosystems).

Plasmid constructs and virus production

sgRNAs (Supplementary Fig. S10A–B) were designed using the CRISPR Design Tool (<http://crispr.mit.edu/>). sgRNA oligos were cloned into the lentiviral vector V2TC, which bicistronically expresses both sgRNAs and mCherry. To validate the targeted mutations, genomic DNA was isolated from the infected organoids, and the T7E1 (Vazyme, EN303-01) assay was performed.

Organoid genome editing and efficiency testing

After dissociation of organoids into single cells using TrypLE, the cells were resuspended in viral supernatant supplemented with 1: 1000 (v/v) polybrene in a 24-well plate, centrifuged at 800 g for 1 h, and then incubated at 37 °C for 2 h. The solution was collected and centrifuged at 400 g for 5 min. The cells were resuspended in ice-cold Matrigel and seeded into 48-well plates for continued culturing. Genomic DNA was extracted from infected organoids and the T7E1 (Vazyme, EN303-01) assay was performed to confirm the targeted mutations.

Organoid growth assay

In the organoid growth assay, 2,000 single cells were cultured in a 96-well plate embedded in 10 μl of Matrigel (with three independent biological replicates in each group). And the number of organoids was counted after 5 days of culture.

Organoid orthotopic transplantation

Organoid fragments were prepared by digestion in TrypLE at 37 °C, followed by mechanical dissociation, centrifugation, and resuspension in 30% Matrigel. Three hundred thousand cells in a 0.075-ml volume were injected into the left lobe of the lung. Body weight was measured every week. At 3, 7, and 11 weeks, three mice were randomly selected and euthanized for subsequent measurement of tumor volume and weight.

Bioluminescence imaging

After orthotopic transplantation of organoids (1.5 × 10⁶ cells in 150 μl 100% Matrigel per injection), mice were periodically imaged to detect the luciferase fluorescence signal intensity using the IVIS Spectrum in vivo imaging system (PerkinElmer). The mice were anesthetized with isoflurane after 250 μl of 15 mg/ml D-luciferin, sodium salt (Bio Vision, #7903-1G) in PBS intraperitoneally injected. Five minutes later, the mice were placed in the instrument, and imaging was initiated.

Micro-CT

Mice were scanned for 340 s under isoflurane anesthesia using a small animal Quantum FX Micro-CT (PerkinElmer) at a resolution of 45 μm and 90 kV with a 160-mA current. Images were acquired and analyzed using PerkinElmer Quantum FX software.

RNA-seq analyses

For the RNA-seq analysis, three technical duplicates were included for each sample. RNA-seq libraries were constructed using an Illumina stranded mRNA sample

preparation kit (NEB, E7770) according to the manufacturer's protocol, and sequencing was performed on an Illumina NovaSeq 6000 sequencing machine with 150-base pair (bp) paired-end reads.

Western blotting

Western blot analyses were performed using standard methods. The following antibodies were used: CD44 (Abcam, ab189524, 1:2,000), CXCL9 (Cloud-clone-crop, PAB928Hu01, 1:2,000), SPP1 (Proteintech, 22,952-1-AP, 1:1,000), p-STAT1 (Santa cruz, sc-8394, 1:2,000), STAT1 (Huabio, ET1612-22, 1:1,000), IFN- γ (CLOUD-CLONE CORP, PAB928Hu01, 1:2,000), CD68 (Abcam, ab283316, 1:1,000) (See Supplementary Representative WB images for the overlay of colorimetric and chemiluminescent images of the original data).

Statistical analysis

All statistical analyses were performed using R (<http://www.rproject.org>), SPSS version 25.0 and Python (<https://www.python.org/>). The detailed statistical methods used for each analysis are described within the figure legends. Differences were investigated using two-sided paired or unpaired Student's t-tests.

Results

Single-cell landscape in GGN- and PSN-LUAD

Nineteen fresh surgical resections of lung tissue samples were obtained from 19 untreated patients with LUAD at the Department of Thoracic Surgery at Hospital of Chengdu University of Traditional Chinese Medicine and Sichuan Cancer Hospital (all samples were unpaired), including four adjacent normal lung tissue samples; three GGN-MIA, three GGN-IAC, and two GGN-MLC samples; and three PSN-MIA, three PSN-IAC, and one PSN-MLC sample (see Fig. 1A, Supplementary Fig. S1 and Supplementary Table S1).

ScRNA-seq techniques were applied to the samples after digestion into single-cell suspensions. A total of 188,594 cells were obtained from 19 lung tissue samples after quality filtering, including 20,145 cells from the GGN-MIA group, 32,233 cells from the GGN-IAC group, 6,539 cells from the GGN-MLC group, 22,273 cells from the PSN-MIA group, 16,184 cells from the PSN-IAC group, 21,235 cells from the PSN-MLC group, and 45,038 cells from the NC group (Supplementary Table S2). Cells were categorized into 12 clusters and annotated with canonical marker gene expression (Fig. 1B), including T cells epithelial cells, B cells, endothelial cells, macrophages, dendritic cells, fibroblasts, mast cells, neutrophils, plasma cells, lymphatic-endothelial, and smooth muscle cell. After annotating the cell types, they were assigned to tumor stages based on the origin of each

cell population (Supplementary Fig. S2). Subsequently, a thorough investigation and summary of the heterogeneity in the composition of various cell types in normal lung tissue (NC group), GGN- and PSN-LUAD tissues were conducted (Supplementary Fig. S3A-D). Unlike the NC group, enrichment of T and B lymphocytes was observed in the PSN-LUAD and GGN-LUAD groups, while myeloid cell infiltration was reduced. Furthermore, as GGN-LUAD and PSN-LUAD undergo changes in invasion and metastasis, differences in the composition of intra-tumoral cells were exhibited in tissue-specific resident populations. These findings suggest that variations in cellular composition at different tumor tissue stages might correlate with mechanisms of disease progression.

Heterogeneity of epithelial cells during the invasion and metastasis of PSN- and GGN-LUAD

The LUAD tumor cells originated from epithelial cells and share many genetic markers with normal epithelial cells [17]. To accurately distinguish tumor cells from normal epithelial cells in the TME, we performed dimensionality reduction and clustering analysis on 31,498 epithelial cells. By integrating information from copy number variations (inferCNV) (Fig. 2A), CopyKat (Fig. 2B), and lineage-specific gene expression (Supplementary Fig. S4A), we successfully distinguished malignant epithelial cells from non-malignant normal epithelial cells. Analysis of non-malignant normal cells identified subpopulations of epithelial cells, including AT1, AT2, basal, ciliated, club, and goblet cells (Fig. 2C).

The heterogeneity of epithelial cells was further investigated. RNA velocity trajectories were constructed by utilizing the identified tumor cells and normal epithelial cell subtypes. The transcriptional states revealed that tumor cells were located between AT1 and AT2 cells (Supplementary Fig. S4B). Subsequently, the characteristics of malignant tumor cells were evaluated using methods such as Cytotrace, CellCycleScoring, and AddModuleScore. The Cytotrace scoring results indicated that the tumor cell population exhibited a stronger lineage differentiation capability (Fig. 2D), and the AddModuleScore results using the Hallmark gene set showed that tumor cells exhibit higher epithelial-mesenchymal transition (EMT) characteristics (Fig. 2E). Moreover, it was observed that the Cytotrace scores and EMT scores at different stages in the PSN-LUAD group were generally higher than those in the GGN-LUAD group, revealing different levels of malignancy between the two groups (Fig. 2F).

Although the Cell-Cycle Scoring results indicated a minimal impact of the cell cycle on the current dataset, a higher proportion of cells in the G2M and S phases was exhibited by the tumor cell population

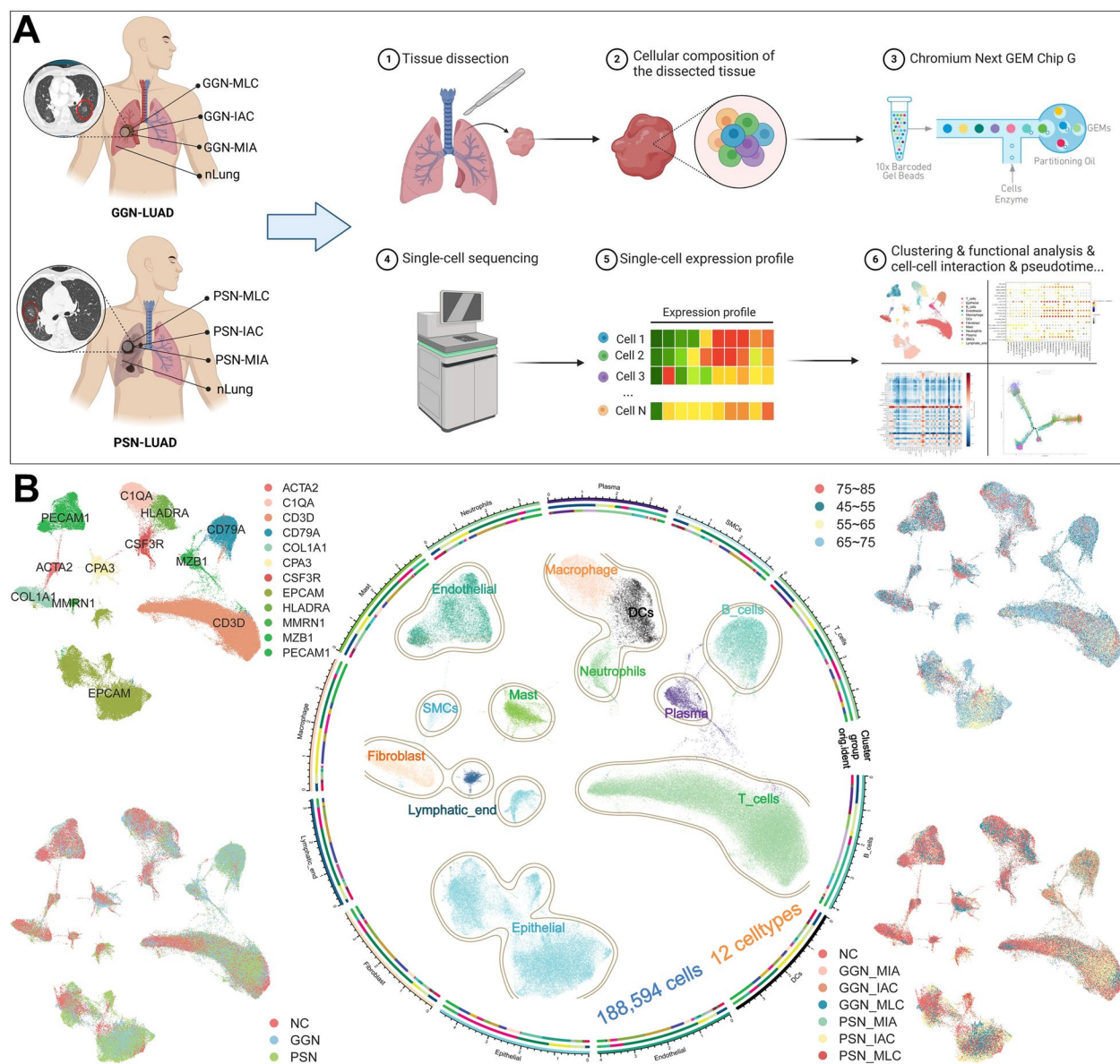


Fig. 1 The radiological features manifest as cellular landscapes within the tumors and adjacent tissues of lung adenocarcinoma (LUAD) patients with ground-glass nodules (GGN) and part-solid nodules (PSN). **A** Study overview: we employed single-cell RNA sequencing to analyse fresh human tissue samples obtained from nineteen patients. This cohort included eight cases of GGN-LUAD cases, comprising three instances of minimally invasive adenocarcinoma (MIA), three cases of invasive adenocarcinoma (IAC), and one cases of metastatic lung cancer (MLC) tissues. Additionally, there were eight cases of PSN-LUAD, with the same distribution of MIA, IAC, and two cases of MLC tissues. Four adjacent normal control (NC) tissues were also included for comprehensive analysis. **B** Uniform Manifold Approximation and Projection (UMAP) visualization was conducted on the 188,594 profiled cells (Centre). Clockwise from the top left, the four corner insets display the UMAP plots of marker gene expression, age distribution, radiographic feature, and grouping by different disease stages

(Supplementary Fig. S4C). It has been shown that Ki-67 is expressed throughout the cell cycle except for the G0 phase, and elevated Ki-67 expression is a marker of higher malignancy [18]. Therefore, Ki-67 was used as a staining marker for the proliferative status of tumor cells. The staining results showed a significant increase in Ki-67 expression as the disease progressed from the

MIA to the IAC stage, with a further marked increase in Ki-67 staining intensity in the MLC stage (Fig. 2G). Additionally, a significant correlation with prognosis in survival analysis was shown by the gene encoding Ki-67, MKI67 (Fig. 2H). The transcriptional program heterogeneity of epithelial cells at different progression stages in GGN- and PSN-LUAD was highlighted by

these data, which may be clinically relevant to disease progression.

Endothelial cells orchestrate angiogenesis

during the invasion and metastasis of PSN- and GGN-LUAD

Endothelial cells are vital cell types constituting the blood/lymphatic vessels, which can influence the nutrient delivery and oxygen supply in the tumor microenvironment [19]. Previous studies have indicated that a reduction in endothelial cells leads to the formation of a hypoxic tumor environment, and hypoxia further promotes tumor cell metastasis by inducing metabolic reprogramming [20]. Our findings align with this hypothesis. Compared to normal tissues, both GGN- and PSN-LUAD exhibit a significant decrease in the proportion of endothelial cells in the TME, with PSN-LUAD showing a lower endothelial cell ratio. Crucially, the MLC stage exhibits a more noticeable decrease in endothelial cell counts than the MIA and IAC stages (Supplementary Fig. 3A).

To further observe the trends in different subtypes of endothelial cells, we subclustered endothelial cells and annotated them into six cell types: Arterial, Capillary, Capillary_Aerocyte, Tip, Vein and Lymphatic cells (Fig. 2I, Supplementary Fig. S4D). By analyzing the proportions of vascular endothelial cell subtypes in the TME, it was found that the proportion of Tip cells in all tumor samples was higher than in normal samples, and this proportion increased gradually with disease progression, while the proportions of other major pulmonary endothelial cell types did not change significantly (Supplementary Fig. S4E). Velocity analysis showed that Tip cells are positioned along the developmental and differentiation trajectory between Vein endothelial cells and Capillary endothelial cells (Fig. 2J), and Cytotrace results indicated that a stronger differentiation capacity is possessed by Tip cells (Fig. 2K). It has been reported that hypoxia levels and vascular growth factor (VEGF) signals

in the microenvironment can be sensed by Tip cells, which are the primary cell type responsible for inducing the formation of the tumor capillary network. Tumors rely on angiogenesis to meet their metabolic demands at different growth stages. In this study, a higher proportion of Tip cells was observed in PSN-LUAD samples compared to GGN-LUAD samples (Supplementary Fig. S4E), which may be one of the potential mechanisms underlying the faster invasion and metastasis observed clinically in PSN-LUAD.

Detailed analysis of myeloid cells revealed an immune microenvironment niche transitions driven

by CXCL9 + TAMs and TREM2 + TAMs

Myeloid cells are key components of the TME [21]. Through cellular subtype proportion analysis, it was evident that myeloid cells significantly increase in proportion during the MLC stage of GGN- and PSN-LUAD (Supplementary Fig. S3A). To pinpoint the myeloid cell crucial for tumor development, the functions of the important cell subclusters were explored. As previously observed, macrophages constituted a major cluster of myeloid cells and played a dominant role (Fig. 3A, Supplementary Fig. S5 A-B). The RNA velocity revealed that, apart from RTM + TAMs, all other TAMs originated from classical monocytes (Fig. 3B), which provides a theoretical direction for studying the trajectory of macrophage differentiation. Additionally, the expression of classical M1 and M2 features in these macrophage subtypes was evaluated using M1 and M2 scoring. The M1 score of CXCL9 + TAMs and the M2 score of TREM2 + TAMs were the highest among the macrophage subgroups (Fig. 3C).

We further analyzed the proportion of TREM2 +, RTM, CXCL9 + TAMs, IL-1 β +, and MMP19 + TAMs subsets during GGN- and PSN-LUAD invasion to explore their distribution characteristics (Fig. 3E). TREM2 + TAMs are crucial for mediating macrophage

(See figure on next page.)

Fig. 2 The expression patterns of epithelial cells and endothelial cells during the invasive and metastatic processes of ground-glass nodules (GGN)- and part-solid nodules (PSN)-lung adenocarcinoma (LUAD). **A** The copy number variation (CNV) analysis results of tumor cells at different stages of GGN-LUAD and PSN-LUAD diseases compared to normal epithelial cells. Colors indicate the $\log_2$ CNV ratio, with red representing amplification and blue representing deletion. **B** The result of the CopyKat algorithm for various types of epithelial cells. The left panel displays Seurat-clusters, where each point corresponds to a cell, and different colors represent different clusters. The right panel shows the identification of aneuploid and diploid epithelial cells. **C** Uniform Manifold Approximation and Projection (UMAP) plot of epithelial cells derived from tumors and tumor-adjacent normal tissues, color-coded by different epithelial cell subtypes. **D** The distribution of the predicted order, as speculated by CytoTRACE, in tumor cells. The color represents the level of cell stemness, with different shades indicating high or low stemness. **E** Activity score of epithelial-mesenchymal transition (EMT) in each cell. **F** The distribution of EMT scores in different stages of GGN- and PSN-LUAD. **G** IHC was employed to evaluate the expression of Ki-67, a LUAD marker, in lung tissues. **H** Kaplan-Meier analysis of overall survival rates in the GEPIA-LUAD cohorts based on expression levels of the Ki-67 gene. **I** UMAP plot illustrating endothelial cells derived from tumors and tumor-adjacent normal tissues, color-coded by different endothelial cell subtypes. **J** RNA velocity analysis reveals the developmental trajectory of the endothelial cell lineage. **K** The distribution of the predicted order, as speculated by CytoTRACE, in endothelial cells. The color represents high or low stemness of the cells

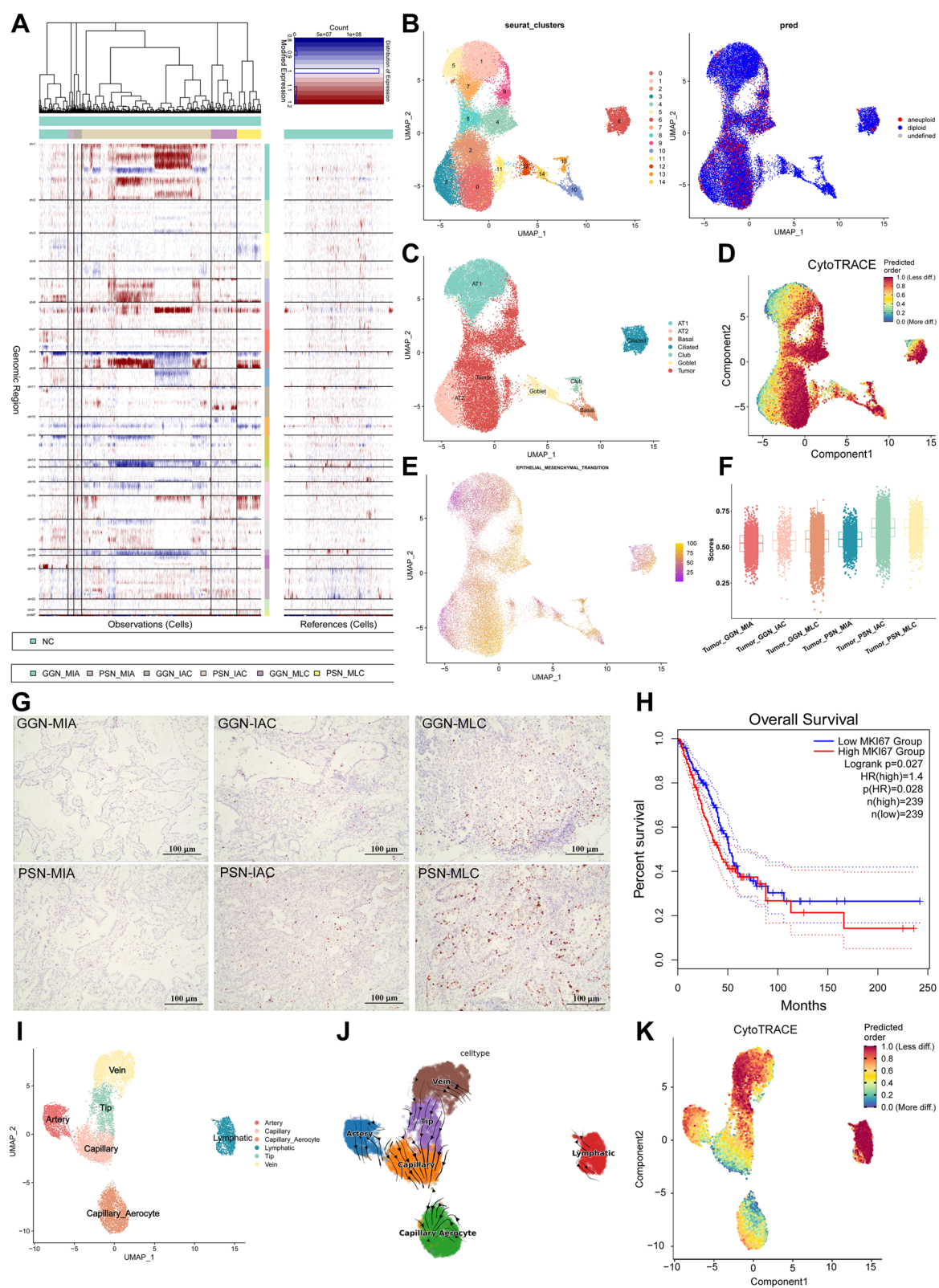


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polarization and promoting escape from lung cancer [22]. A higher percentage of TREM2+ TAMs was observed in GGN- and PSN-LUAD than in normal samples at different disease stages, with a higher percentage in PSN-LUAD than in GGN-LUAD. Moreover, higher RTM+ TAMs enrichment was found in GGN-LUAD compared to PSN-LUAD. CXCL9-secreting TAMs exhibit anti-tumor activity [23]. Compared to normal tissues, an increased proportion of CXCL9+ TAMs was found in GGN- and PSN-LUAD tissues. However, based on the distribution of different macrophage subtypes, CXCL9+ TAMs were present in the lowest proportion and significantly decreased during the MLC period. These results suggest a potential antagonistic relationship between TREM2+ and CXCL9+ TAMs. To further validate this hypothesis, multiplex immunofluorescence staining was used to analyze tumor sections from different stages of GGN and PSN-LUAD. The distribution patterns of TREM2+ and CXCL9+ TAMs in the tumor microenvironment confirmed the potential antagonistic relationship between these two TAM subtypes (Fig. 3D).

We employed Monocle2 to analyze the differentiation trajectories of classical monocytes, CXCL9+ TAMs, and TREM2+ TAMs during the process of invasion and metastasis in LUAD. At different stages of GGN- and PSN-LUAD, the trajectory trees of CXCL9+ TAMs showed consistent differentiation (Fig. 3F, Supplementary Fig. S5C), indicating that CXCL9+ TAMs exhibited the same function at different stages of LUAD progression. Pseudotime analysis results showed that classical monocytes exhibited multiple tree nodes and different differentiation trajectories, among which monocytes in the state 5 differentiated into CXCL9+ TAMs (Supplementary Fig. S5D). CXCLs were significantly upregulated during the differentiation of monocytes into CXCL9+ TAMs, with CXCL9 showing the most significant upregulation (Fig. 3G).

Additionally, genes related to CXCL9+ TAMs differentiation were highly associated with immune regulation. In the CXCL9+ TAMs developmental trajectory, genes related to antigen presentation (HLA-DPA1, HLA-DRA, and HLA-DRB1) gradually increased in expression, whereas pro-inflammatory genes (IL-1B, S100A12, S100A8, and S100A9) decreased (Supplementary Fig. S5E). The enrichment analysis results of the genes related to CXCL9+ TAM differentiation trend showed that the top 50 genes were primarily enriched in T cell differentiation, antigen presentation, and inflammatory response (Supplementary Fig. S5F-G).

To investigate the potential impact of macrophages expressing TREM2 during cancer progression, we conducted pseudotime analysis on TREM2+ TAMs (Fig. 3H, Supplementary Fig. S6A). The findings reveal cellular differentiation heterogeneity in TREM2+ TAMs, highlighting the elevated expression of the SPP1 gene in TREM2+ TAMs at states 4 and 7 (Fig. 3I, Supplementary Fig. S6B).

To discern the differentiation inclination of TREM2+ TAMs, we quantified the cellular-level variations of TREM2+ /SPP1+ TAMs and TREM2+ /SPP1- TAMs across distinct disease stages of LUAD (Fig. 3J). The findings elucidate that during the MIA-IAC stages of LUAD, TREM2+ TAMs exhibited a predilection toward manifesting SPP1-characteristics, whereas during the LUAD-MLC stage, an escalation in the proportion of SPP1+ TAMs occurred. The proportion of SPP1+ TAMs in PSN-MLC was significantly higher than in GGN-MLC. Notably, we observed that the proportions of CXCL9+ TAMs and TREM2+ SPP1+ TAMs exhibited opposite trends across different stages of LUAD (Supplementary Fig. S6C).

Subsequent differential gene expression analysis of TREM2+ /SPP1+ TAMs and TREM2+ /SPP1- TAMs was conducted to explore the underlying

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Fig. 3 Detailed analysis of myeloid cells. **A** Uniform Manifold Approximation and Projection (UMAP) plot illustrating subsets of myeloid cells derived from tumors and adjacent normal tissues, color-coded to show different macrophage cell subtypes. Each dot represents a cell, colored to indicate cluster origin. **B** The RNA velocity analysis reveals the developmental trajectory of the macrophage lineage within the myeloid cell population. **C** The mean score of the M1 or M2 signature across TREM2+ tumor-associated macrophages (TAMs), RTM+ TAMs, CXCL9+ TAMs, IL1B+ TAMs, and MMP19+ TAMs. **D** Immunofluorescence showing the expression of CXCL9 and TREM2 in the tumor microenvironment (TME) at different stages of ground-glass nodules (GGN)-lung adenocarcinoma (LUAD) and part-solid nodules (PSN)-LUAD. Representative images display CXCL9 in yellow, TREM2 in green, CD68 as a macrophage marker in red, and nuclei counterstained with DAPI in blue. The scale bar is 20 μ m. **E** Analysis of the proportion of subsets of macrophages cell types in normal control (NC), GGN-minimally invasive adenocarcinoma (MIA), GGN-invasive adenocarcinoma (IAC), GGN-metastatic lung cancer (MLC), PSN-MIA, PSN-IAC, and PSN-MLC. **F** Diagram illustrating the inferred CXCL9+ TAM cell differentiation pathway generated by Monocle. **G** The trajectory of four typical genes (CXCL10, CXCL11, CXCL16, CXCL9). **H** Pseudotime trajectory of LUAD cell state transitions. **I** The differentiation trajectory of TREM2+ TAMs can be categorized into SPP1- TAMs (TREM2+ /SPP1- TAMs) and SPP1+ TAMs (TREM2+ /SPP1+ TAMs). **J** Analysis of the proportion of SPP1+ TAMs and SPP1- TAMs cell type in NC, GGN-MIA, GGN-IAC, GGN-MLC, PSN-MIA, PSN-IAC and PSN-MLC. **K** The volcano plot illustrates the significantly differentially expressed functional genes between SPP1+ TAMs and SPP1- TAMs. **L** Representative multiplex immunohistochemistry staining images of mononuclear cells, TREM2+ SPP1+ TAMs, and TREM2+ SPP1- TAMs in formalin-fixed, paraffin-embedded tumor tissues from LUAD patients. The scale bar is 50 μ m

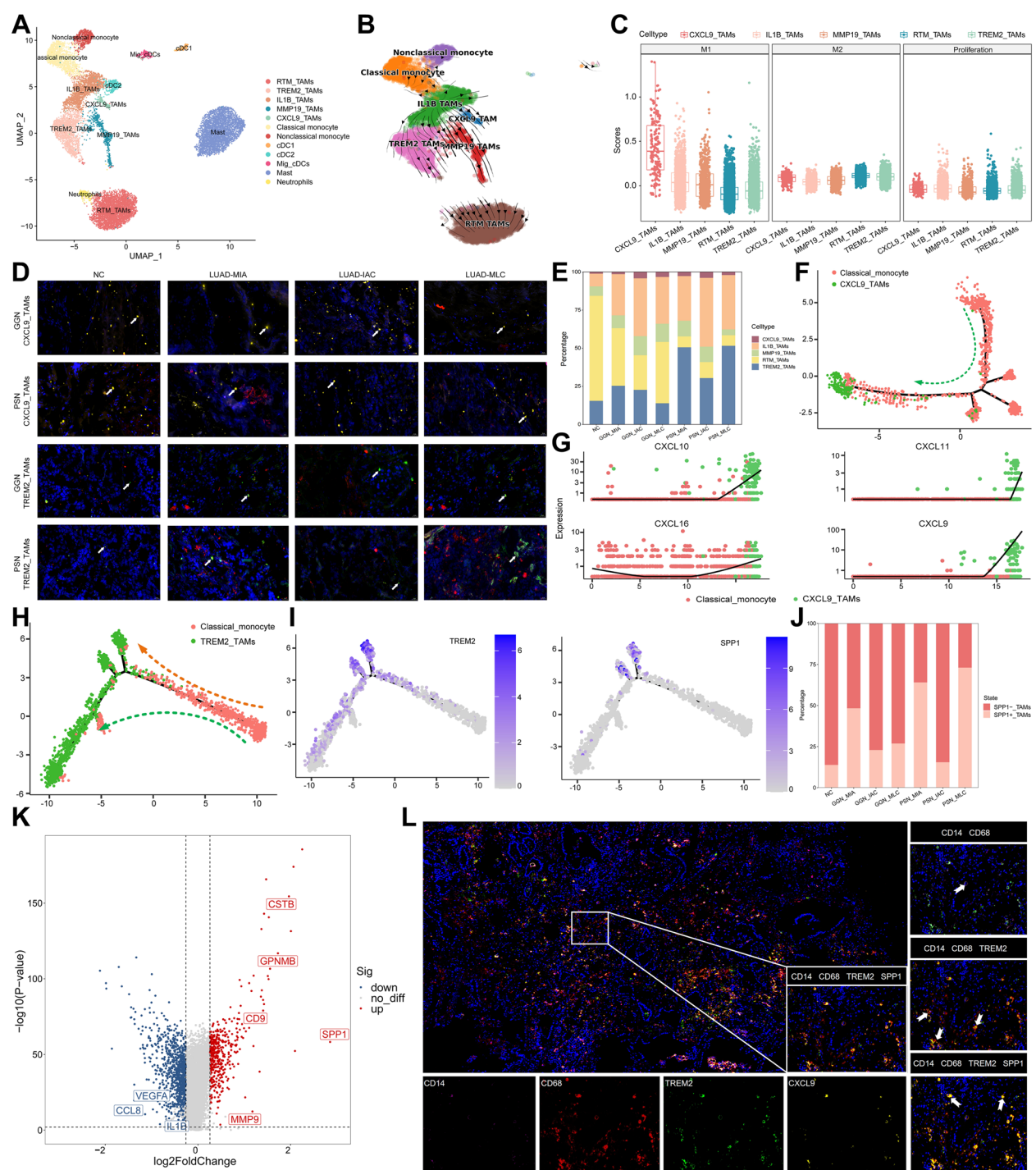


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factors contributing to the differentiation heterogeneity of TREM2+ TAMs (Supplementary Fig. S6D). The results reveal elevated expression of CCL8 and IL1B genes in TREM2+/SPP1- TAMs. This suggests a potential regulation of TREM2+/SPP1-TAMs by the hypoxic micro-environment [24]. Additionally, we observed significant

upregulation in the VEGFA gene in TREM2+/SPP1-TAMs. Enrichment analysis indicated a strong correlation in gene expression with pathways related to angiogenesis (Supplementary Fig. S6E). In the advanced stages of LUAD infiltration, TREM2+ TAMs predominantly exhibited SPP1+ characteristics. Concurrently,

differential gene analysis revealed the upregulation of genes such as SPP1, CSTB, GPNMB, CD9, and MPP9 in TREM2+/SPP1+ TAMs (Fig. 3K).

Given the difficulty in detecting the differentiation trends of macrophages in tissues, the differentiation states of macrophages were analyzed using marker genes specific to different states. The presence of TREM2+/SPP1+ TAMs and TREM2+/SPP1- TAMs in tumor tissues was revealed by multiplex immunofluorescence results. The expression patterns of these marker genes support the speculation about the differentiation pathways of TREM2+ TAMs and further validate the accuracy of pseudotime trajectory analysis (Fig. 3L). In summary, these results indicate that alterations in CXCL9+ TAMs and TREM2+ TAMs have a significant impact on the evolution and progression of LUAD and may explain the differing developmental trends between PSN-LUAD and GGN-LUAD.

Changes in the infiltration characteristics of lymphoid cells in the immune microenvironment

To reveal the TME infiltration of T cells during the invasive and metastatic processes of GGN-LUAD and PSN-LUAD, we compared the changes in T cell proportions across NC, GGN-MIA, GGN-IAC, GGN-MLC, PSN-MIA, PSN-IAC, and PSN-MLC. Compared to NC, there was a significant increase in T cell proportions during the GGN-MIA, GGN-IAC, and PSN-MIA stages. Notably, a significant decrease in T cell infiltration was observed in the PSN-IAC group compared to the PSN-MIA group, with T cell proportions lower than in the NC group (Supplementary Fig. S3A, Supplementary Fig. S7A). Thereafter, we identified twelve sub-clusters of T cells: CD4+MT, CD4+Tfh, CD4+Tn, CD4+Treg, CD4+Tstr, CD8+GNLY_Teff, CD8+GZMK_Teff, CD8+Trm, Classical NK, ISG+T, mucosal associated invariant T cells, and nonclassical NK according to the expression of canonical lineage markers (Fig. 4A-B) [25]. Compared with NC, the infiltration frequency of CD4+T cells, which are adaptive immune cells, increased in GGN- and PSN-LUAD, with the highest level observed in the IAC stage (Supplementary Fig. S7A C-D). In addition, we observed a persistent increase in the proportion

of CD8+Trm cells in GGN-LUAD and PSN-LUAD, with a higher proportion in the GGN-MLC stage than the PSN-MLC stage (Fig. 4C). CD8+Trm cells are capable of continuously monitoring the tumor microenvironment, identifying and eliminating newly emerging tumor cells or small residual tumors, which helps prevent tumor recurrence and progression.

Beyond T cells, we have also focused on another lymphocyte lineage cell type—B cells. The infiltration of B cells in GGN-LUAD significantly increased during the MIA-IAC stage and decreased in the MLC stage. Similarly, in PSN-LUAD, there was a notable increase in the proportion of B cells during the MIA stage, and a significant decrease in the IAC and MLC stages (Supplementary Fig. S7B). Subsequently, B cells were subdivided into three clusters: naive B cells, memory B cells, and plasma cells (Fig. 4D-E). Through the analysis of the proportions of three B cell subtypes, we did not find significant differences in the phenotypic proportions between different groups (Fig. 4F), indicating that the changes in the immune microenvironment dominated by B cells were related to the overall infiltration abundance of B cells in the tissue. These findings suggest that macrophages and T cells play major roles in immune heterogeneity during GGN- and PSN-LUAD invasion and metastasis.

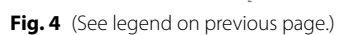
In addition, the results from the external single-cell transcriptomics data were highly consistent with the immune cell types detected in our study, and our research captured immune cell subtypes that were not identified in previous studies, such as RTM+TAMs, MMP19+TAMs, CD4+MT, and CD4+Tstr (Supplementary Fig. S8A-F). These results validate the reliability of the immune cell subtype identification in our study.

Cell-cell interactions indicate different patterns of tumor invasion and metastasis between GGN- and PSN-LUAD

To delve deeper into the heterogeneity of the immune microenvironment in GGN- and PSN-LUAD at different disease stages, we utilized CellPhoneDB to analyze and compare the interaction strengths among different immune cell types (measured by the sum of mean values of intercellular signaling communication), revealing differences in immune cell activity across the

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Fig. 4 **A** and **D** Uniform Manifold Approximation and Projection (UMAP) plot illustrating subsets of T cells (**A**) and B cells (**D**) derived from tumors and adjacent normal tissues, color-coded by different macrophage cell subtypes. Each dot represents a single cell, with color indicating its cluster origin. **B** and **E** Heat maps demonstrate the expression levels of specific cell markers in subsets of T cell subset (**B**), and B cell subset (**E**). Each row represents an individual cell marker, and each column represents a specific cell subset. **C** and **F** Analysis of the proportion of T cell (**C**) and B cell subsets (**F**) in normal control (NC), ground-glass nodules (GGN)-minimally invasive adenocarcinoma (MIA), GGN-invasive adenocarcinoma (IAC), GGN-metastatic lung cancer (MLC), part-solid nodules (PSN)-MIA, PSN-IAC, and PSN-MLC. **G-L** Circos plots depicting intercellular CXCL signaling between all cell types in GGN-LUAD and PSN-LUAD, including GGN-MIA (**G**), GGN-IAC (**H**), GGN-MLC (**I**), PSN-MIA (**J**), PSN-IAC (**K**), and PSN-MLC (**L**)



MIA–IAC–MLC stages in both GGN and PSN–LUAD. Specifically, during the MIA stage of GGN–LUAD, active immune cells were primarily B cell and T cell subpopulations. As tumor infiltration progressed, the signaling communication intensity between these subpopulations and other myeloid cells gradually increased. However, during the MLC stage, the signaling communication between all immune cell types and B cell and T cell subpopulations significantly weakened, with the active cell types in the tumor immune microenvironment transitioning to macrophage subpopulations. In the corresponding stages of PSN–LUAD, we similarly observed a transition from an immune microenvironment dominated by T and B cell subpopulations to one dominated by macrophages, indicating a close association between this transition and tumor disease progression. Compared to GGN–LUAD, this transition occurred earlier in PSN–LUAD, possibly reflecting the faster progression of PSN–LUAD (Supplementary Table S3, Supplementary Fig. S9A–F).

We further explored the causes of this transition. Utilizing CellChat, we conducted an in-depth analysis of the key roles of T-cell, B-cell, and macrophage subpopulations in the progression and metastasis of PSN–LUAD and GGN–LUAD (Fig. 4G–L). We observed a significant enhancement of intercellular interactions related to the CXCL signaling pathway during the GGN–MIA and GGN–IAC stages (Fig. 4G and H). This phenomenon was also validated during the MIA–IAC stage of PSN–LUAD (Fig. 4J and K). Additionally, we observed a decrease in the activity of the CXCL pathway in both the GGN–MLC and PSN–MLC groups at the MLC stage. However, there was an enhancement of SPP1 pathway activity specifically during the PSN–MLC stage (Fig. 4I and L). Our research indicated that the SPP1 signal received by tumor cells mainly originates from cells other than CXCL9+TAMs, such as TREM2+TAMs and RTM+TAMs, with TREM2+TAMs being the highest proportion of monocyte-derived macrophages during the PSN–MLC stage. This is consistent with the latest findings in *Science*,

mutual exclusion in the expression of CXCL9 and SPP1 in TAMs [26].

IFN- γ regulates the interaction between CXCL9+TAMs and CD8+Trm cells, affecting the progression of GGN– and PSN–LUAD invasion and metastasis

Subsequently, we explored the important mechanisms of signal transduction between CXCL9+TAMs and TREM2+TAMs during GGN– and PSN–LUAD invasion and metastasis. Pathways such as INTERFERON_GAMMA_RESPONSE (IFN- γ), INFLAMMATORY_RESPONSE, and P53_PATHWAY were more active in CXCL9+TAMs. Pathways such as ANGIOGENESIS, EPITHELIAL_MESENCHYMAL_TRANSITION, and NOTCH_SIGNALING were more active in TREM2+TAMs (Fig. 5A). Interferon (IFN)- γ is an important macrophage activator that induces the secretion of chemokines, attracting and activating other immune cells to the tumor site, thereby enhancing tumor immune infiltration and inflammatory responses [27]. The scoring results of the IFN- γ response pathway activity indicate that it exhibits higher activity in CXCL9+TAMs (Fig. 5B). Compared with different GGN–LUAD stages, the IFN- γ signaling pathway in PSN–LUAD showed lower activity. We assessed the source of IFN- γ signaling during GGN– and PSN–LUAD invasion and metastasis based on the analysis results of Cellchat (Fig. 4G–L). During GGN–LUAD invasion, IFN- γ was released by various immune cells: CD8+Trm cells, CD8+GZMK_Teff, CD8+GNLY_Teff, and Classical_NK cells (Fig. 4G–I). During PSN–LUAD invasion, IFN- γ was only released by NK and CD8+Trm cells (Fig. 4J–L). This accounts for the weakened interaction between CXCL9+TAMs and CD8+Trm cells in the invasive stage of PSN–LUAD compared to GGN–LUAD.

To further elucidate the influence of IFN- γ on the differentiation of CXCL9+TAMs, we performed an intersection analysis of IFN- γ -responsive genes, high-activity transcription factors regulating CXCL9 expression, and genes associated with the sequential differentiation

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Fig. 5 **A** The heatmap displays Gene Set Variation Analysis (GSVA) enrichment scores for CXCL9+ tumor-associated macrophages (TAMs) and TREM2+TAMs cell characteristic pathways across different disease stages of lung adenocarcinoma (LUAD). **B** The boxplot shows the activity scores of all TAMs IFN- γ responsive modules based on the added module score. **C** Heatmap showing the transcription factor activity regulation for all immune cells from different disease stage samples of LUAD using SCENIC. **D** The Venn diagram illustrates the intersection genes among genes associated with CXCL9+TAMs differentiation, IFN- γ pathway-related genes, and specific transcription factors targeting CXCL9. **E** The ridge plot displays the expression of STAT1 in different TAMs. The height of the ridges represents abundance, and the horizontal axis reflects the gene expression levels. **F** Line graphs and heat maps depict the expression level variations in STAT1 and CXCL9 genes at different disease stages of ground-glass nodule (GGN)–LUAD and part-solid nodule (PSN)–LUAD. **G** A pie chart displays the correlation coefficients of STAT1 and CXCL9 gene expression levels. **H** Heatmap showing changes in the activity of the top 5 transcription factors during the differentiation process of SPP1– and SPP1+TAMs. **I** Ranking of regulons in classical monocyte, TREM2+SPP1-TAMs, and TREM2+SPP1-TAMs based on RSS. **J** Volcano plot showing the top 10 differentially expressed genes between SPP1+TAMs and SPP1–TAMs ($\log_2$ fold change ≥ 0.5 , $p \leq 5 \times 10^{-2}$)



differentiation of CXCL9+TAMs. Additionally, STAT1 exhibited higher expression levels in CXCL9+TAMs compared to other TAMs (Fig. 5E). We corroborated the regulatory relationship between STAT1 and CXCL9 by

conducting a correlation analysis of their expression levels at various stages of tumor development. The results revealed a correlation coefficient of 0.902 between the two, indicating a similar expression trend (Fig. 5F). In the analysis of different differentiation stages (SPP1+ and SPP1-) of TREM2+ TAMs (Fig. 5G), transcription factors such as NFATC1(+), EGR3(+), KLF6(+), and MAF(+) exhibited high activity expression during the TREM2+/SPP1- TAMs differentiation stage. Combining the DEG analysis of TREM2+/SPP1+ TAMs and TREM2+/SPP1- TAMs, we identified EGR1, FOSB, and STAT3 as significantly upregulated in TREM2+/SPP1- TAMs (Fig. 5I). In addition, we found that BHLHE41(+) and NR1H3(+) can directly regulate the expression of SPP1, playing a key role in inducing TAMs to develop towards the SPP1+ phenotype (Fig. 5H).

In vivo validation of the role of the IFN- γ /STAT1 pathway in the development and progression of LUAD

To simulate the pathological progression of human LUAD, primary and orthotopic LUAD models were constructed in immunocompetent mice using organoid culture and CRISPR-Cas9 gene editing technology. cBioPortal for Cancer Genomics (<http://cbioportal.org>) provides a resource for exploring, visualizing, and analyzing multidimensional cancer genomics data. We analyzed 1,590 primary LUAD samples and identified the top mutated genes in LUAD, with *TP53* (47%) and *Kras*^{G12D} (36%) ranking as the top two cancer genes. AT2 cells were isolated from the lung tissue of CAG-Cas9-EGFP mice and cultured to form lung organoids. sgRNAs and cDNAs were then introduced into these organoids to knock out and overexpress genes associated with human LUAD (*Trp53*^{-/-}; *Kras*^{G12D}) (Fig. 6A, Supplementary Fig. S10A-B). Normal lung epithelial organoids (WT) were used as a control, and WT, *Trp53*^{-/-}, and *Trp53*^{-/-}; *Kras*^{G12D} (TK) organoids were cultured in 3D Matrigel. The organoids in the TK group were mCherry positive (Supplementary Fig. S10C), and the efficiency of sg*Trp53* was confirmed by a T7E1 assay (Supplementary Fig. S10D). The overexpression of *Kras* was verified using the qPCR and Integrative Genomics Viewer (IGV) (Supplementary Fig. S10E). Additionally, bright-field images showed that WT organoids exhibited well-organized cystic structures, *Trp53*^{-/-} organoids displayed multilayered epithelial compositions, and TK organoids formed disorganized solid masses (Fig. 6B, D-F). Manual counting and Ki-67 staining revealed that TK organoids had a higher proliferation rate (Fig. 6C).

The genetically edited TK organoids were transplanted into the lungs of mice and monitored using luciferase live imaging and micro-computed tomography (micro-CT) (Fig. 7A-C, Supplementary Fig. S10F). Biopsy

showed specific green fluorescent protein (GFP) expression in a single lesion at the injection site, indicating that the tumor originated from the transplanted organoids (Fig. 7D). Results of hematoxylin and eosin (H&E) staining and IHC demonstrated early LUAD development at 3 weeks, progression to LUAD by 7 weeks, and a higher degree of malignancy by 11 weeks (Fig. 7D-G). RNA-seq results from tumor tissues at 3, 7, and 11 weeks in mice indicated a gradual decrease in immune cell infiltration as LUAD developed and progressed (Fig. 7H-J). This finding is consistent with the results of Single-cell transcriptomics, suggesting that immune cell exhaustion occurs in the tumor microenvironment as the disease advances. The Western blot results at 3, 7, and 11 weeks showed that the protein expression levels of IFN- γ , STAT1, p-STAT1, CXCL9, and CD8 gradually decreased, while the protein expression levels of SPP1 and CD44 gradually increased. Additionally, ELISA validation was performed on the serum of mice at the three stages. These results are consistent with the data from single-cell transcriptomics, indicating the reliability of the single-cell transcriptomics results.

Discussion

Distinct imaging features of LUAD manifest as unique growth patterns that are intricately linked to patient prognosis. Although ongoing studies have examined changes in the TME of various radiological components of LUAD [12, 13], thorough analyses of the heterogeneity of the TME and disease prognosis regulations for GGN- and PSN-LUAD progression are significantly lacking. The outcomes of this study revealed distinctive TME patterns between GGN- and PSN-LUAD, identifying CXCL9+ TAMs and TREM2+ TAMs as crucial TME subsets influencing the ecological differences in invasion and metastasis between the two radiographic phenotypes. We further delineated a highly coordinated network of pro- or anti-tumor variables comprising CXCL9+ TAMs and TREM2+ TAMs, exploring their potential sources, interactions with T and tumor cells, and expression patterns of relevant regulatory genes in different radiographic subtypes of LUAD (Fig. 8). We posit that these findings enhance our understanding of their heterogeneity during GGN- and PSN-LUAD occurrence and progression, offering potential therapeutic strategies worthy of further validation.

The TME, primarily comprising neoplastic cells and myriad stromal cells, undergoes specific alterations in cell types that are intricately linked to tumor progression [28]. We identified 12 distinct cell types, representing different stages of GGN- and PSN-LUAD (MIA, IAC, and MLC). Our study revealed the heterogeneity in lineage differentiation features, proliferative characteristics, and

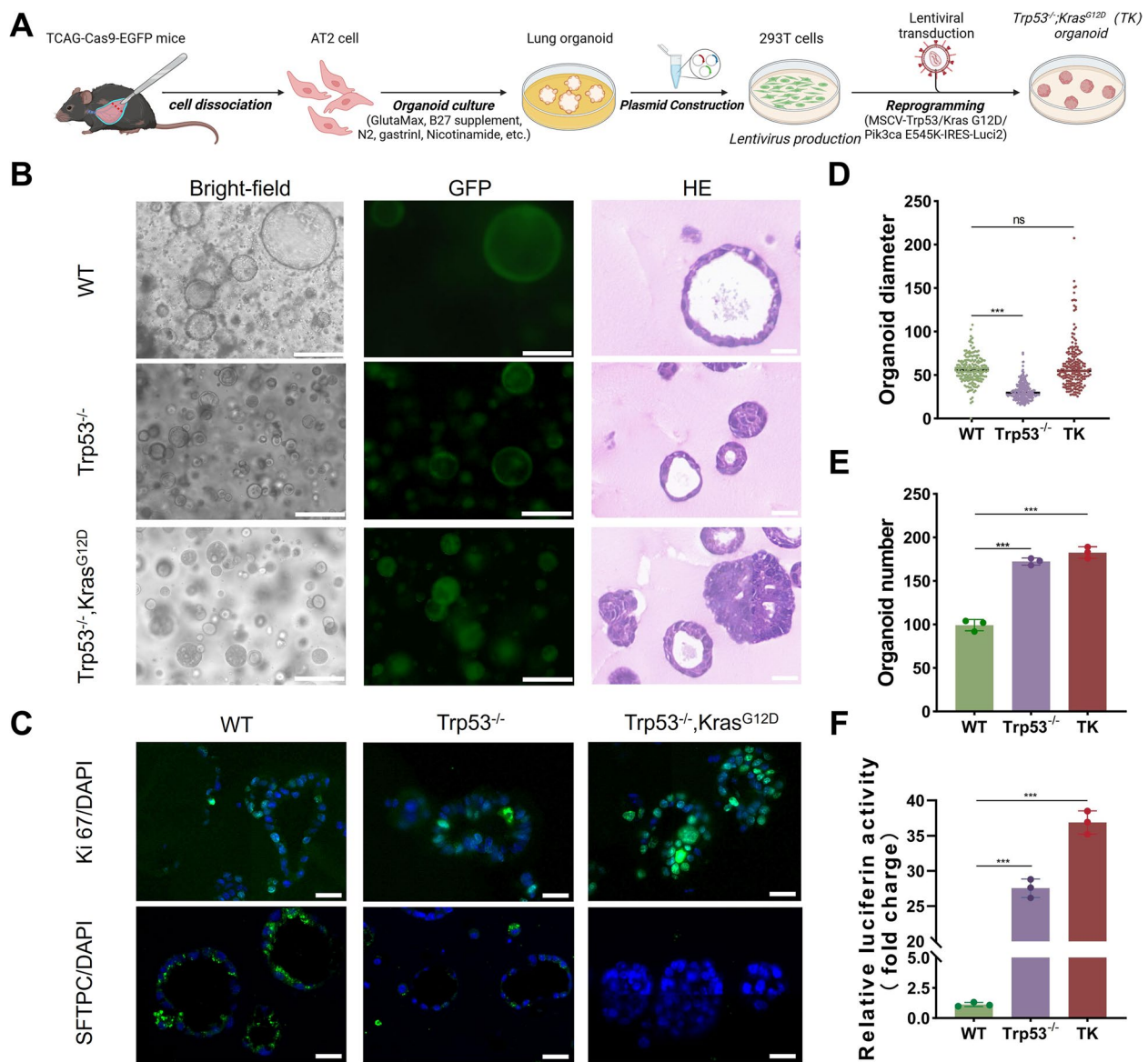


Fig. 6 The generation of $Trp53^{-/-}; Kras^{G12D}$ drivers in premalignant lung organoids. **A** Stepwise induction protocol for TK ($Trp53^{-/-}; Kras^{G12D}$) organoids derived from lung tissue of mice. **B** Representative bright-field (right) images and GFP (middle), and H&E (left) in WT, $Trp53^{-/-}$, TK mouse lung organoids. Scale bars: 200 μ m (right), 200 μ m (middle), and 20 μ m (left). **C** Representative immunofluorescence staining of Ki-67 (top) and SFTPC (bottom) in WT, $Trp53^{-/-}$, and TK mouse lung organoids. Scale bar: 20 μ m. **D** The number of WT ($n=3$), $Trp53^{-/-}$ ($n=3$), and TK ($n=3$) organoids. Data are presented as the means $\pm$ SEM; p values were calculated using unpaired t test. *** $p < 0.001$. **E** The diameters of WT, $Trp53^{-/-}$, and TK organoids. Data are presented as the means $\pm$ SEM; p values were calculated by unpaired t test. *** $p < 0.001$. **F** Luminescence signal intensity of WT ($n=3$), $Trp53^{-/-}$ ($n=3$), and TK ($n=3$) lung organoids. Data are presented as the means $\pm$ SEM; p values were calculated by unpaired t test. *** $p < 0.001$

EMT among cancer cells from different stages of GGN and PSN-LUAD. Related studies have shown that these malignant features provide protection and support for tumor initiation and are involved in tumor progression [29]. Our research indicates that, during the progression of GGN and PSN-LUAD, the reduction of endothelial cells induces the formation of a hypoxic TME. The

hypoxic environment further promotes the generation of Tip cells, influencing angiogenesis in the new stages. Immune cells are crucial for determining the biological behavior of cancer cells. We identified diverse immune cell subpopulations based on their phenotypes and analyzed variations in their fractions within the TME. Notably, substantial differences in the allocation of immune

cell subpopulations between GGN- and PSN-LUAD during invasive metastasis were discovered. This supports the notion that considerable heterogeneity exists in LUAD within the TME. In both GGN- and PSN-LUAD, a consistent phenomenon of gradual depletion of CD8+T and NK cells was observed. These cells can directly eliminate tumor cells during LUAD invasion and metastasis, but their gradual depletion may lead to tumor immune evasion [30, 31]. This finding aligns with a previous study that demonstrated a reduction in T-cell activity among patients with LUAD. Macrophages are the dominant myeloid cell population in LUAD, with heterogeneous subpopulations prevalent during GGN- and PSN-LUAD invasion and metastasis. Lower levels of macrophage subpopulation infiltration were exhibited in PSN-LUAD compared to GGN-LUAD. This is consistent with previous research findings indicating that macrophage depletion may promote tumor development [32].

The TME is an intricately interdependent ecosystem in which dynamic interactions among immune cells regulate cancer onset and progression [33]. Robust immune reactions within tumor tissues indicate the concerted effort of the human body to resist cancer cell infiltration and impede tumor growth. In our study, the interaction intensity among immune cells at different GGN-LUAD stages significantly surpassed that of PSN-LUAD, suggesting a heightened susceptibility of PSN-LUAD to immune escape compared with GGN-LUAD. Notably, CXCL9+TAMs and TREM2+TAMs, compared with other macrophage subsets, exhibited substantial differences in their interactions with T and tumor cells during LUAD invasion and metastasis, with distinct imaging features. Although the precise mechanisms through which CXCL9+TAMs modulate LUAD pathogenesis remain unclear, emerging data suggest that TAMs expressing CXCL9 are novel contributors to anticancer immunity [26]. CXCL9+TAMs secrete the chemokine CXCL9, which binds to the chemokine receptor CXCR3 on the surface of CD8+Trm cells, guiding

and attracting CD8+Trm cells to tumor sites to exert anti-tumor effects [34]. Our research indicates that GGN-LUAD exhibited a more robust signaling interaction between CXCL9+TAMs and CD8+Trm than PSN-LUAD. However, in the MLC stage of both LUAD types, the signaling interaction between CXCL9+TAMs and CD8+Trm cells was significantly weakened, suggesting that it is a crucial factor in LUAD metastasis. Heightened TREM2+TAMs expression has been documented in various malignancies, including NSCLC and hepatocellular carcinoma [35, 36]. TREM2-deficient mice reportedly exhibit weakened tumor growth in several transplant models, accompanied by an augmented response against PD1. TREM2+TAMs facilitate the growth and dissemination of cancer cells upon binding to CD44 receptors on the surface of tumor cells, contributing to the formation of an immunosuppressive TME [37]. Our investigation revealed that during the PSN-MLC stage, TREM2+TAMs exhibited robust binding to CD44 receptors on the surface of tumor cells, displaying intensified interactions. This phenomenon may significantly contribute to the accelerated metastasis observed in PSN-LUAD compared with that in GGN-LUAD. Notably, high infiltration of CXCL9+TAMs was accompanied by TREM2+TAMs infiltration, suggesting a possible antagonistic effect between CXCL9+TAMs and TREM2+TAMs.

We further explored the potential mechanisms underlying the disparities in interaction signals between CXCL9+TAMs and CD8+Trm cells in GGN- and PSN-LUAD. Notably, across different GGN- and PSN-LUAD stages, the IFN- γ signaling pathway exhibited heightened activity in the gene enrichment analysis of CXCL9+TAMs. IFN- γ is involved in activating cellular immunity and subsequently stimulating anti-tumor immune responses. According to Ding, IFN- γ can trigger CXCL9 expression, which attracts cytotoxic T lymphocytes to the cancerous tissue [38]. STAT1 is a pivotal mediator of IFN- γ -induced gene activation [39]. We observed a gradual decrease in IFN- γ levels secreted by

(See figure on next page.)

Fig. 7 Modeling lung adenocarcinoma (LUAD) of different stages using engineered lung organoids in mice. **A** A diagram showing the method of detailed orthotopic transplantation. **B** Representative bioluminescent images of the TK mice at 3, 7, and 11 weeks. **C** Photograph of representative TK tumors at 3 ($n=4$), 7 ($n=4$), and 11 weeks ($n=4$) weeks. Scale bar: 5 mm. **D** Representative bright-field (left) and green fluorescent (right) images of TK tumors at 3, 7, and 11 weeks. Scale bar: 4 mm. **E** Representative H&E, TTF-1, CK7, Ki-67, and Cas9 staining of TK tumors at 3, 7, and 11 weeks. Scale bar: 50 μ m. **F** Statistical graph showing the weight of the control and TK groups during 12 weeks. Data are shown as means $\pm$ SD, calculated using two-sided Student's t-test. **G** Statistical graph showing the volume of TK tumors at 3, 7, and 11 weeks ($n=4$). **H** Heatmap showing the expression levels of diagnostic and metastatic of LUAD marker genes in TK tumor tissues at 3 ($n=3$), 7 ($n=3$), and 11 weeks ($n=3$). **I** Gene Ontology enrichment plot of upregulated genes in the TK mice tumor tissues, comparing the TK tumor tissues at 3, 7, and 11 weeks. **J** Heatmap of the proportion of six types of immune cells and functions. **K** Western blot analysis of STAT1, p-STAT1, IFN- γ , CXCL9, CXCR3, SPP1, and CD44 in TK tumor samples at 3, 7, and 11 weeks. **L-N** ELISA measurement of IL-1 β , TNF- α , GZMB in the serum from TK tumor samples at 3, 7, and 11 weeks. Data are presented as the means $\pm$ SEM; p values were calculated using unpaired t test. ** $p < 0.01$

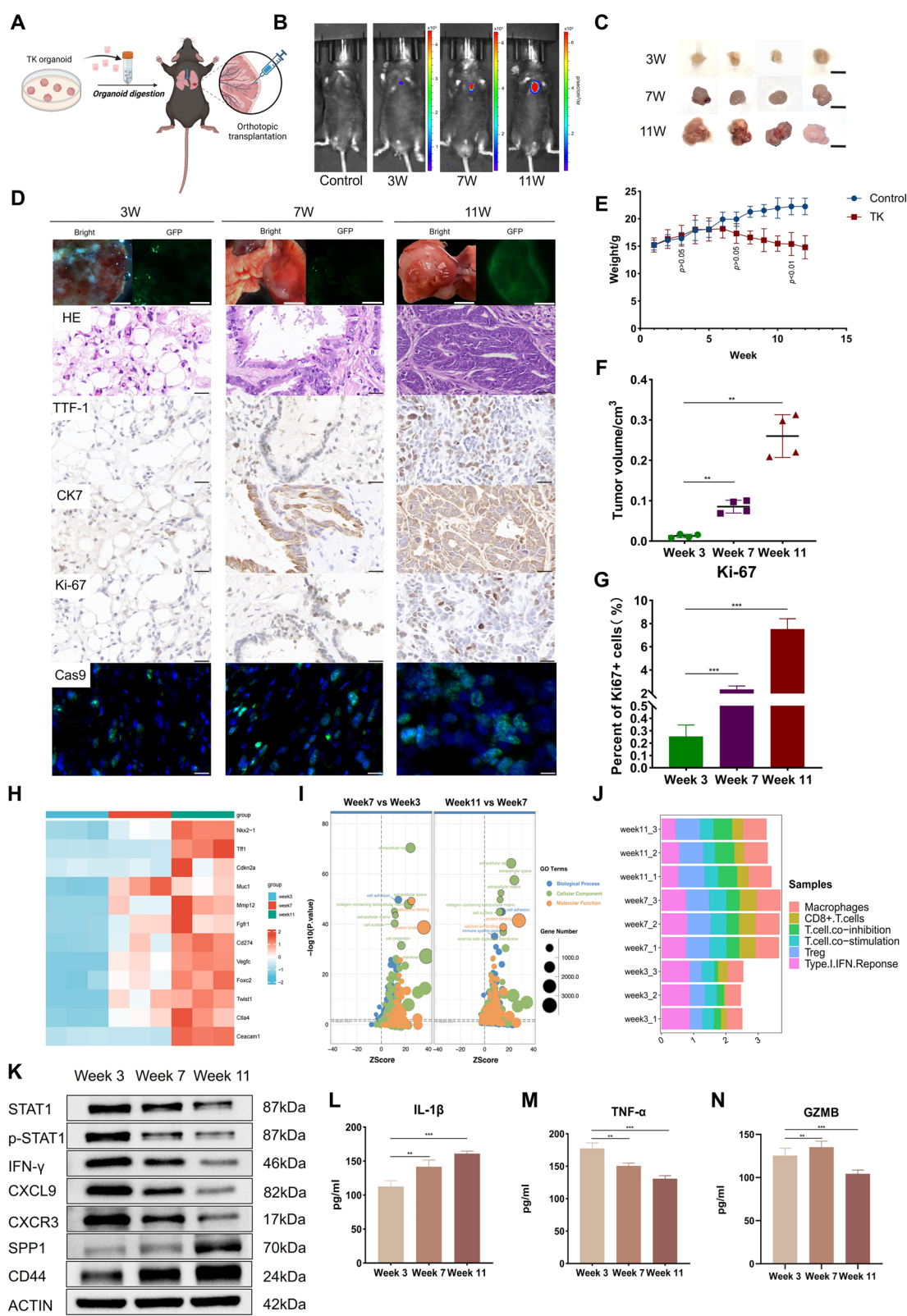


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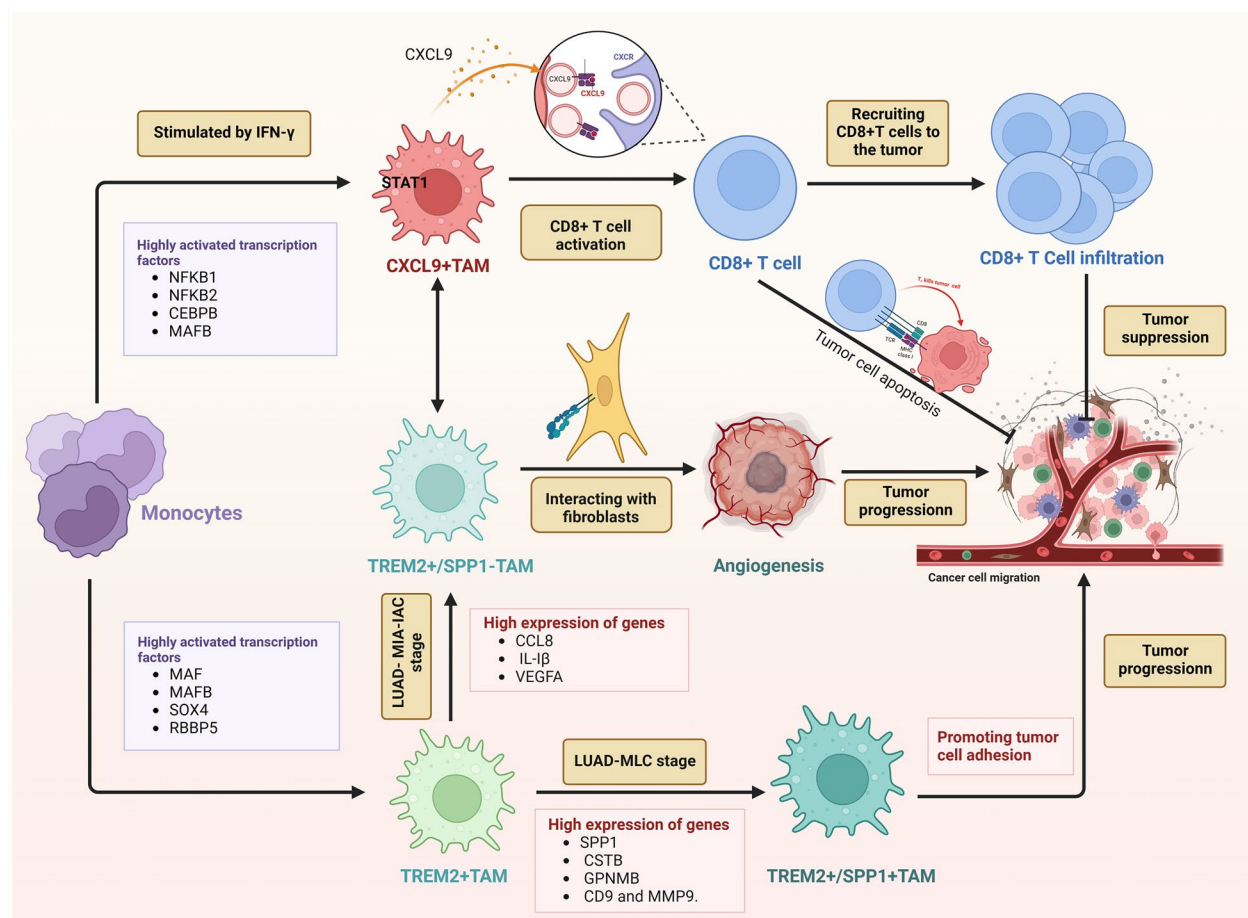


Fig. 8 Mechanism schema diagram. Inhibition of IFN- γ can promote the transformation of monocyte into CXCL9+TAMs through the transcription factor STAT1 pathway, thus reshaping the tumor immune microenvironment, especially enhancing the expression of CD8+T cells

immune cells as GGN- and PSN-LUAD progressed, leading to a corresponding decrease in STAT1 activation. Under such circumstances, the receptor-ligand interaction between CXCL9+TAMs and CD8+T cells also diminished. This aligns with recent research outcomes regarding IFN- γ [40]. Injecting high and low doses of IFN- γ can reportedly promote tumor regression and tumor metastasis, respectively [41]. Notably, GGN-LUAD exhibits a stronger IFN- γ signaling pathway than PSN-LUAD, activating STAT1 to regulate CXCL9 secretion in TAMs, thereby enhancing cell communication with its homologous receptor, CXCR3. The results presented above revealed that IFN- γ could influence GGN- and PSN-LUAD invasion and metastasis by activating STAT1 signaling and modulating the interaction between CXCL9+TAMs and CD8+T cells.

To validate the role of the IFN- γ /STAT1 signaling axis in the development and progression of LUAD, this study established a robust and mature mouse model based on organoid culture and CRISPR/Cas9 technology to

simulate the process of LUAD development. A key advantage of the combination of organoid culture and CRISPR/Cas9 technology is the high degree of control over tumor development [42]. This uniformity in the process of knocking out tumor suppressor genes and introducing oncogenes into organoids ensures that the impact of each genetically modified organoid on tumor development remains consistent and a high degree of reproducibility in tumor development across individuals [43]. Therefore, we were able to capture the entire process of tumorigenesis of TK organoids in mice and observed that tumor development followed a specific temporal pattern, with different phenotypic and pathological characteristics appearing at 3, 7, and 11 weeks, respectively. Pathological results showed that at week 3, the lesion presented with atypical epithelial cell proliferation, prominent nucleoli, and tightly packed cell arrangement; at week 6, the cell morphology showed epithelial thickening, deep staining of nucleoli, weak positivity for Thyroid Transcription Factor (TTF), weak positivity for Ki-67, and positivity for

CK-7; by week 10, the cell morphology was varied, with enlarged nucleoli, deep nuclear staining, strong positivity for TTF, strong positivity for Ki-67, and a significant increase in malignancy. These pathological features closely resemble those of human LUAD, confirming the effectiveness of this model in simulating the development of human LUAD. Then, we performed transcriptome sequencing on the tumor tissues of LUAD mice at 3, 7, and 11 weeks, confirming the phenomenon of immune cell exhaustion as LUAD disease progresses. Additionally, Western blotting results demonstrated changes in the IFN- γ /STAT1 signaling axis during the development and progression of LUAD, thereby confirming the results of scRNA-seq analysis.

This study has certain limitations. First, we grouped samples based on distinct disease stages (MIA/IAC/MLC) in GGN- and PSN-LUAD. However, due to insufficient samples received for the PSN-MLC stage, cells in this group could not undergo significant statistical analysis at the cellular level compared to cells in other groups. In the future, we will collect an adequate number of samples to further confirm the reliability of our conclusions. Consequently, we performed a comparative analysis of the divergent outcomes to scrutinize the distinctions between the invasive metastases of GGN- and PSN-LUAD. Second, an exhaustive depiction of all cell types, subtypes, and phenotypes within a single report may not be feasible. Hence, our study highlights key observational outcomes that will be investigated more comprehensively in the future. Despite these limitations, this study provides novel biological insights into the heterogeneity of LUAD, characterized by diverse radiological features, potentially influencing the advancement of precision medicine in clinical LUAD research.

Conclusions

This study confirmed significant heterogeneity in the cellular composition and immune responses in GGN- and PSN-LUAD, and further revealed the differential enrichment and interaction patterns of CXCL9+ TAMs and TREM2+ TAMs, as well as their regulatory genes, during different stages of LUAD progression. Our findings suggest that IFN- γ influenced GGN- and PSN-LUAD invasion and metastasis by activating STAT1 signaling and modulating the interaction between CXCL9+ TAMs and CD8+ Trm.

Abbreviations

LUAD	Lung adenocarcinoma
GGN	Ground-glass nodule
PSN	Part-solid nodule
TME	Tumor microenvironment
scRNA-seq	Single-cell RNA sequencing
AIS	Adenocarcinoma in situ
MIA	Minimally invasive adenocarcinoma

IAC	Invasive adenocarcinoma
MLC	Metastatic lung cancer
NSCLC	Non-small cell lung cancer
TAM	Tumor-associated macrophage
IRBs	Institutional Review Boards
cDNA	Complementary DNA
PCR	Polymerase Chain Reaction
UMI	Unique molecular identifiers
UMAP	Uniform Manifold Approximation and Projection
MCMC	Markov chain Monte Carlo
DEG	Differentially Expressed Genes
GSVA	Gene Set Variation Analysis
GO	Gene Ontology
CNV	Copy number variation
CT	Computed tomography
NC	Normal control
EMT	Epithelial-mesenchymal transition
VEGF	Vascular growth factor
MT	Memory T cells
Tfh	T follicular helper cells
Tn	T naive cells
Treg	Regulatory T cells
Tstr	T stromal cells
Teff	T effector cells
Trm	Tissue-resident memory T cells
ELISA	Enzyme-Linked Immunosorbent Assay
TTF	Thyroid Transcription Factor

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12943-024-02177-7>.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4: Figure S1. The imaging performance of the GGN-LUAD patients and PSN-LUAD patients. Figure S2. UMAP visualization was performed on the cells of 19 samples, color-coded by different cell subtypes. Figure S3. A. The proportion of each cell type was analyzed in normal control (NC), ground-glass nodules (GGN)-lung adenocarcinoma (LUAD) and part-solid nodules (PSN)-LUAD, as well as in NC, GGN-minimally invasive adenocarcinoma (MIA), GGN-invasive adenocarcinoma (IAC), GGN-metastatic lung cancer (MLC), PSN-MIA, PSN-IAC and PSN-MLC. B. Cellular composition analysis of twelve cell types among NC, GGN-LUAD, and PSN-LUAD patient groups. C. Cellular composition analysis of twelve cell types among NC, GGN-MIA, GGN-IAC, and GGN-MLC patient groups. D. Cellular composition analysis of twelve cell types among NC, PSN-MIA, PSN-IAC, and PSN-MLC patient groups. The lower hinge, middle line, and upper hinge of boxplots represented the first, second, and third quartiles of the distributions. The upper and lower whiskers corresponded to the largest and smallest data points within the 1.5 interquartile range, with all actual data values plotted as dots adjacent to the boxplots. Figure S4. A. The heatmap displays the expression levels of specific cell markers within epithelial cell subpopulations. Each row represents an individual cell marker, while each column represents a specific cell subpopulation. B. The RNA velocity analysis reveals the developmental trajectory of the macrophage lineage within the epithelial cell population. C. The activity of cell cycle score for each cell. D. The heatmap displays the expression levels of specific cell markers within epithelial cell subpopulations. Each row represents an individual cell marker, while each column represents a specific cell subpopulation. E. The proportion of vascular-associated endothelial cells was analyzed in normal control (NC), GGN-minimally invasive adenocarcinoma (MIA), GGN-invasive adenocarcinoma (IAC), GGN-metastatic lung cancer (MLC), PSN-MIA, PSN-IAC and PSN-MLC. Figure S5. A. Heatmaps demonstrate the expression levels of specific cell markers in myeloid cell subsets. B. The proportion of myeloid cell subsets was analyzed in normal control (NC), GGN-minimally invasive adenocarcinoma (MIA), GGN-invasive adenocarcinoma (IAC),

GGN–metastatic lung cancer (MLC), PSN–MIA, PSN–IAC, and PSN–MLC. The Y-axis represents the ratio of the target cell count to the total cell count within each group (%), with groups represented by different colors. C. Diagram illustrating the inferred CXCL9+ tumor-associated macrophages (TAMs) cell differentiation pathway by Monocle. Pseudotime is shown colored in a gradient from dark to light blue, and start of pseudotime was indicated. D. Illustrates the development trajectory of different samples. E. The cellular evolutionary trajectory of CXCL9+ TAMs: Top 50 Differentiation-Related Gene Expressions and GO Enrichment. Genes (rows) were clustered into three modules, and cells (columns) were ordered according to pseudotime. Red indicates high expression, while blue indicates low expression. F–G. KEGG (F) and GO (G) annotations of genes associated with the differentiation trend of CXCL9+ TAMs are shown. The dot size represents the number of genes, and the color scale represents the adjusted *p* value. Figure S6. A. An illustration of the inferred differentiation pathway of TREM2+ tumor-associated macrophages (TAMs) based on Monocle analysis is shown. Pseudotime is visually represented by a gradient from dark to light blue, indicating the initiation of pseudotime. The developmental starting point is on the right, and the developmental endpoint is on the left. B. The developmental trajectory of different samples is demonstrated. C. The proportion of each TAM cell type was analyzed in normal controls (NC), GGN–minimally invasive adenocarcinoma (MIA), GGN–invasive adenocarcinoma (IAC), GGN–metastatic lung cancer (MLC), PSN–MIA, PSN–IAC, and PSN–MLC. D. The pseudotime heatmap displays the involvement of relevant genes in the differentiation process of TREM2+ TAMs. Based on their expression trends, the clusters with the largest changes along the pseudotime trajectory were divided into three clusters, and the representative processes for each cluster were shown. E. GO annotation of genes associated with the differentiation trend of TREM2+ TAMs is shown. The dot size represents the number of genes, and the color scale represents the adjusted *p* value. Figure S7. A–D: Bar plots of the percentages of T cells (A), B cells (B), T cell subsets cells (C–D) type among normal control (NC), ground-glass nodules (GGN)–lung adenocarcinoma (LUAD) and part-solid nodules (PSN)–LUAD groups. The Y-axis represents the ratio of the target cell count to the total cell count within each group (%). Groups are shown in different colors. The error bars represent each group's $\pm$ standard error of the mean. We used the t-test to calculate the significance of differences in cell proportions between the experimental groups. **p* < 0.05; ***p* < 0.01; ****p* < 0.001. Figure S8. External single-cell transcriptome data validation confirms the annotation and clustering of macrophage, T cell, and B cell subpopulations. A, C and E. UMAP plots illustrating subsets of myeloid cells (A), T cells (C) and B cells (E) derived from tumors and tumor-adjacent normal tissues, color-coded by different macrophage cell subtypes. Each dot represents a single cell, with the color indicating the cluster origin. B, D and F: Heatmaps demonstrate the expression levels of specific cell markers in subsets of myeloid cell (B), T cell subsets (D), and B cell subsets (F). Figure S9. A–F. Comparative analysis of cell–cell interactions between normal control (NC) and ground-glass nodules (GGN)–minimally invasive adenocarcinoma (MIA) (A), GGN–MIA and GGN–invasive adenocarcinoma (IAC) (B), GGN–IAC and GGN–metastatic lung cancer (MLC) (C), NC and part-solid nodules (PSN)–MIA (D), PSN–MIA and PSN–IAC (E), PSN–IAC and PSN–MLC (F). The total occurrences of interactions were calculated for each specific cell pair within the mentioned comparisons. A deeper red color indicates a higher interaction intensity. Figure S10. A. A schematic representation of sgRNA (top) and Kras cDNA (bottom) expression plasmids is shown. B. Gene sequencing peak maps of Kras (top) and Trp53 (bottom) in the plasmid are shown. The box indicates the 12th amino acid and its codon in the Kras gene, as well as the Cas9 target sequence of Trp53. C. Fluorescence images of plasmid transfection in 293T cells (24h) and mouse lung organoids (48h) transduced by viral vectors are shown. Scale bar: 200 μ m. D. T7 endonuclease 1 (T7E1) assays were performed on Trp53 using the infected organoids. E. Relative expression levels of Kras in WT, Trp53^{-/-} and TK organoids were measured by real-time quantitative PCR. Data are shown as mean $\pm$ SD. The significance was calculated using a two-sided Student's t-test. F. The Integrative Genomics Viewer displays the mutation site of Kras in TK organoids. The box indicates the 12th amino acid and its codon in the Kras gene. G. Representative micro-CT

images of TK mice models at 3, 7, and 11 weeks are shown. The circled area indicates the tumor regions.

Supplementary Material 5.

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Not applicable.

Authors' contributions

YFR, QM and XZ: Conceptualization, Writing—original draft. YFR, QM, CXH, JLR, FL, JJT and JWH: Formal analysis, Methodology, Software, Visualization. JWH, YZ, SYT, HJ, LFZ, HZL, PX and XZ: Data curation, Resources. YFR, QM, PW, LTY, WS and XF: Investigation, Validation. CZ and FMY: Conceptualization, Funding acquisition, Writing—review & editing. FMY: Project administration, Supervision.

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Data availability

The datasets generated and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request. The external single-cell transcriptome data were obtained from the Genome Sequence Archive (GSA) at the BIG Data Center, Beijing Institute of Genomics (BIG), with the accession number HRA001130.

Declarations

Ethics approval and consent to participate

This study adhered to the principles of the Declaration of Helsinki and was approved by the Institutional Review Boards of the Chengdu University of Traditional Chinese Medicine Affiliated Hospital (Institutional Review Board No. 2022KL-051) and Sichuan Cancer Hospital (Institutional Review Board No. SCCHEC-02–2022-109). Written informed consent was obtained from all patients prior to the surgery. Animal experiments were approved by the Institutional Animal Care and Use Committees of Hospital of Chengdu University of Traditional Chinese Medicine.

Consent for publication

We have obtained consents to publish this paper from all the participants of this study.

Competing interests

The authors declare no competing interests.

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