

SPP1⁺ Senescent Macrophages Promote Tumor Microenvironment Remodeling and Facilitate the Progression of Early-Stage Lung Adenocarcinoma with Mixed Ground-Glass Nodules

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Abstract

The majority of persistent ground-glass nodules (GGNs) are ultimately identified as early-stage lung adenocarcinoma (LUAD). Investigating the molecular mechanisms driving the malignant progression of GGNs could support the development of preventive and therapeutic approaches to block the development and progression of early LUAD. Macrophages (Macs) play a key role in establishing an immunosuppressive tumor microenvironment (TME). Yet, their specific contribution to the progression of early-stage LUAD presenting as mixed ground-glass nodules (mGGNs) remains unclear. Through single-cell RNA sequencing (scRNA-seq) of normal lung tissue, ground-glass areas, and solid components from mGGNs, along with validation via multicolor immunohistochemistry (mIHC) and flow cytometry, we observed elevated levels and accumulation around tumors of immunosuppressive SPP1⁺ alveolar macrophages and monocyte-derived macrophages (Mo-Macs), particularly the latter. This buildup at the tumor edge may hinder immune cell infiltration into the tumor interior, thereby facilitating the malignant shift of GGNs. SPP1⁺ Macs exhibit a senescent profile while also showing the ability to promote tumor metastasis and new blood vessel formation. Analysis of clinical LUAD tissue microarrays and the TCGA-LUAD dataset indicated that a higher proportion of tumoral SPP1⁺ Macs correlates with worse patient outcomes. Moreover, SPP1⁺ Macs contribute to an immunosuppressive TME by interacting with other immune populations. Experiments in cell cultures and animal models demonstrated that knocking out SPP1 suppressed the immunosuppressive polarization and senescence in Macs, restored a less suppressive TME, and slowed the expansion of LUAD xenograft tumors. These results suggest a novel therapeutic avenue targeting SPP1⁺ Macs to potentially slow or prevent the conversion of GGNs into early-stage LUAD.

Keywords: Lung adenocarcinoma, Mixed ground-glass nodule (mGGN), Macrophage, Sphingosine-1-phosphate phosphatase 1 (SPP1), Tumor microenvironment

Introduction

The widespread use of low-dose computed tomography (LD-CT) for lung cancer screening has led to more frequent detection of lung adenocarcinoma (LUAD)

through the appearance of pulmonary nodules (PNs) [1, 2]. Among these, pure ground-glass nodules (pGGNs) represent a distinct category characterized by relatively slow growth and a favorable prognosis [3]. Nevertheless, many persistent pGGNs evolve into mixed ground-glass nodules (mGGNs) or even solid pulmonary nodules (SPNs), and are eventually confirmed as early-stage LUAD [4, 5]. However, the precise cellular and molecular processes underlying this progression are not fully understood, leaving no established clinical methods to prevent the malignant transformation in these nodules. Notably, mGGNs contain both ground-glass and solid elements, providing an important model for studying

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Received: 15 April 2024; Accepted: 31 August 2024

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How to cite this article: Anderson M, Wong L, Lee S. SPP1⁺ Senescent Macrophages Promote Tumor Microenvironment Remodeling and Facilitate the Progression of Early-Stage Lung Adenocarcinoma with Mixed Ground-Glass Nodules. Arch Int J Cancer Allied Sci. 2024;4(2):186-211. <https://doi.org/10.51847/NcJVhmtsOy>

nodule transformation and the early phases of LUAD initiation and growth [6]. Although earlier research has examined single-cell profiles of PNs [7, 8], these efforts have not thoroughly investigated TME development or the emergence of “pro-tumorigenic niches” specifically in mGGNs.

Tumor-associated macrophages (TAMs) that exhibit immunosuppressive traits are among the most abundant immune cell types in the tumor niche. Research over the past 10 years highlights the importance of these adaptable macrophages (Macs) in cancer initiation, malignant progression, and metastasis [9]. Macrophages generally originate from two main sources: bone marrow-derived monocytes and tissue-resident populations originating from embryonic tissues such as the fetal liver and yolk sac [10]. In non-small cell lung cancer (NSCLC), macrophage populations undergo dynamic shifts as disease progresses, with alveolar macrophages (AMs) declining and monocyte-derived macrophages (Mo-Macs) increasing [11]. In particular, AMs undergo phenotypic changes and spatial reorganization during lung cancer development. These AMs promote the subsequent recruitment of Mo-Macs [12, 13], jointly contributing to the establishment of pro-tumorigenic niches [14]. Consequently, understanding the distinct alterations in key macrophage subsets during mGGN progression and targeting them could represent a promising approach to disrupt suppressive TME formation and halt the shift from pGGN to early LUAD. Current studies on macrophages in lung cancer mainly address later, non-nodular stages [15-17], while research on macrophages in early-stage LUAD with PNs has largely been confined to single-cell sequencing analyses [8, 18, 19] without functional validation through cellular or in vivo models.

In this study, we performed a detailed examination of macrophage subpopulation dynamics and the development of immunosuppressive barriers in early LUAD. This involved comparing single-cell transcriptomic data from matched samples of mGGN patients and confirming the observations with multicolor immunohistochemistry (mIHC), flow cytometry, and experiments using SPP1-knockout mice.

Materials and Methods

Human specimens

The study protocol received approval from the Ethics Committee of Xinqiao Hospital of Army (Third Military

Medical University (No. 145-01) and adhered to the principles outlined in the Declaration of Helsinki. Written informed consent was secured from every participant. Four patients with mGGNs were included based on these criteria: (1) CT scans showing mixed nodules larger than 2 cm; (2) confirmed pathological diagnosis of LUAD; (3) no prior treatment; (4) absence of other malignancies; (6) undergoing surgical resection; (7) willingness to provide consent. Patients with serious comorbidities such as severe COPD, coronary heart disease, or cerebrovascular conditions that precluded surgery were excluded. Matched ground-glass regions (GGRs), solid regions (SRs), and normal lung tissues were obtained from each patient (totaling 12 samples) based on radiographic and histological distinctions between ground-glass and solid areas within the mGGN [20]. Normal lung tissue was harvested approximately 5 cm distant from the malignant mGGN lesion. An additional 15 mGGN specimens were collected for flow cytometry using the same selection criteria. A LUAD tissue microarray comprising 98 patients and 180 samples was obtained from Outdo Biotech (Shanghai, China).

Sample preparation and single-cell RNA-sequencing

Following surgical removal, tissue samples were finely chopped and placed in DMEM (ThermoFisher) supplemented with 0.5% collagenase I/IV (Roche, Basel, Switzerland), 20 U/μl DNase I (Sigma), and 1 mg/ml neutral protease (Invitrogen). The mixture was incubated at 37 °C for 15–30 min with gentle agitation every 5 min. PBS (pH 7.4) was then added, and the suspension was passed through Scienceware Flowmi 70-μm cell strainers. After centrifugation (300g, 4 °C, 5 min), cells were resuspended in 1 mL red blood cell lysis buffer (Sigma), incubated at 25 °C for 2 min, centrifuged again, and resuspended in 400 μg/1 mL BSA-PBS solution before filtering through Scienceware Flowmi 40-μm cell strainers (VWR) using low-retention filter tips (Mettler-Toledo). Viable cell counts were determined with a TC20™ Automated Cell Counter (Bio-Rad). All samples were processed within 3 hours of resection and analyzed according to the manufacturer’s protocol (10X Genomics). Sequencing was performed on a NovaSeq 6000 (Illumina) by Gene Denovo Biotechnology (Guangzhou, China). Raw data were processed with Cell Ranger 3.1.0 to generate count matrices, aligned to the GRCh38 reference genome in R 3.5.3, and analyzed using Seurat v3.0.1.

Unsupervised dimensional reduction and clustering of single cells

Seurat (R 3.0.1) was employed for quality control (QC) and cell filtering. Gene expression matrices for individual samples were normalized using the “NormalizedData” function, then scaled to account for UMI counts and mitochondrial content via the “ScaleData” function (negative binomial regression). Cells were excluded if they exceeded 60,000 UMIs, had fewer than 200 or more than 7,000 detected genes, or showed over 20%/50% mitochondrial UMIs. Batch effects were corrected using Canonical Correlation Analysis (CCA). The “RunTSNE” function was applied for visualization and clustering at varying resolutions (0.5 for overall cells, alveolar cells, fibroblasts, and myeloid cells; 0.6 for T cells; 1.5 for endothelial and B cells). Differentially expressed genes (DEGs) were identified with the Wilcoxon rank-sum test via “FindAllMarkers,” requiring an average log fold change ≥ 0.25 and Bonferroni-adjusted $P < 0.01$. Clusters were further confirmed by mIHC and flow cytometry on mGGM samples.

Gene Ontology and KEGG enrichment analysis

Marker genes for clusters, group DEGs, and stemness-related genes were subjected to functional annotation using the Gene Ontology database (RRID: SCR_002811) and KEGG database (RRID: SCR_018145) in R 3.5.3. Pathways with FDR-corrected $P < 0.05$ were considered significantly enriched.

Gene set variation analysis (GSVA) and gene set enrichment analysis (GSEA)

GSVA was performed with the GSVA R package (1.34.0; RRID: SCR_021058) and GSEA (version 4.0.3; RRID: SCR_003199) on MSigDB gene sets using default settings. Pathway activity differences between cell groups were assessed via generalized linear models. Significantly altered pathways were identified across cell populations. Standard parameters were used for subsequent GSEA analyses.

Contribution evaluation across subsets

The contribution of each cell subset was quantified based on population changes and transcriptional shifts, following previously described methods [21]. Signature genes for alveolar, T, B, SPP1⁺ Macs, and FABP4⁺ Macs were derived from the top upregulated genes comparing Normal to GGR and GGR to SR groups via bulk differential expression analysis. DEGs between

Normal/GGR and GGR/SR in all subsets were identified, with the top-100 DEGs serving as subset-specific signatures. Contribution scores were calculated as the average fold-change of these signature genes within each cluster.

Survival analysis

LUAD transcriptomic and clinical data were downloaded from UCSC. Survival analysis included 510 LUAD patients overall and 284 stage I cases. Kaplan–Meier curves were generated using the R “survival” package and visualized with “survminer::ggsurvplot.” The log-rank test evaluated differences between curves.

mIHC

Formalin-fixed paraffin-embedded specimens from our institution were used, supplemented by a LUAD tissue array from Shanghai Outdo Biotech. Primary antibodies included anti-CD68 (M0876, Dako, RRID: AB_2074844), anti-CD163 (ab74604, Abcam, RRID: AB_1280790), anti-SPP1 (MA5-17180, Invitrogen, RRID: AB_2538651), anti-CD3 (IR503, Agilent), anti-CD8 (MA5-14548, Invitrogen, RRID: AB_10984334), anti-CD56 (MA5-11563, Invitrogen, RRID: AB_10985168), anti-FOXP3 (14-4776-82, Invitrogen, RRID: AB_467554), anti-SFTPC (ab90716, Abcam, RRID: AB_10674024), and anti-CD20 (PA5-16701, Invitrogen, RRID: AB_10980806). Detection was performed using the Opal 7-Color Manual IHC Kit (PerkinElmer, NEL811001KT) according to the manufacturer’s instructions. Slides were coverslipped with Prolong Gold Antifade. Imaging was performed using the Mantra Quantitative Pathology Workstation (PerkinElmer). Single-channel data were acquired with the TissueFAXS system (TissueGnostics Asia Pacific Limited, Austria) and analyzed via StrataQuest (v7.0.1.165). DAPI-stained nuclei were identified, and intensity thresholds were defined to identify positive cell populations for each marker. Percentages of CD68⁺, CD68⁺SPP1⁺, CD68⁺CD163⁺, and CD68⁺CD163⁺SPP1⁺ cells were quantified relative to total nucleated cells.

Trajectory inference of alveolar and CD8⁺ T cell states

A gene-by-cell expression matrix derived from the four distinct CD8⁺ T cell clusters previously identified in Seurat (R 3.0.1) was input for trajectory modeling. Pseudotime reconstruction was executed using Monocle (R package version 2.6.4). After performing dimensionality reduction and arranging cells in order

along the trajectory with the settings $\sigma = 0.001$, $\lambda = \text{NULL}$, $\text{param. } \gamma = 10$, and $\text{tol} = 0.001$, the underlying transcriptional progression was mapped. Genes playing pivotal roles in cell state transitions were identified using an FDR threshold of $1e-5$ and clustered based on their characteristic expression dynamics.

Gene regulatory network inference

Reconstruction of transcription factor (TF) networks relied on the SCENIC pipeline (R 1.1.2). Motif enrichment analysis and network assembly were achieved through RcisTarget (v1.6.0) and GENIE3 (v1.8.0), scanning a collection of 20,000 motifs. For each cell, regulon activity was scored via AUCell (R 1.12.0) using AUC calculations. Activity patterns across cells were subsequently visualized as a clustered heatmap.

Tissue distribution analysis

Tissue enrichment patterns for SPP1⁺ macrophages, FABP4⁺ macrophages, and principal cell populations were quantified by computing observed-versus-expected (Ro/e) cell ratios in normal lung tissue and GGR samples [22]. Expected counts were modeled with the chi-square test.

Cell-cell interaction network construction

Intercellular signaling was explored using CellPhoneDB. Ligand-receptor (L/R) pairs present in over 25% of cells per subset were retained. Interaction networks were depicted as circular diagrams and heatmaps, highlighting specific L/R pairs and their interaction intensities. Statistically enriched L/R pairs between cell populations were identified, with corresponding p-values displayed using the pheatmap function (version 1.0.12, RRID: SCR_016418).

Preparation and culture of bone marrow-derived macrophages (BMDMs)

SPP1-knockout mice on the C57BL/6NCy-Spp1em1/Cy background were obtained from Cyagen Biosciences (Suzhou, China); age-matched wild-type (WT) littermates served as controls. Femurs and tibias were dissected, epiphyses excised, and placed in 15-ml tubes. After allowing large debris to settle for 15 minutes, bone marrow cells were isolated by centrifugation. Then 1×10^7 bone marrow cells were plated per 10-cm dish and differentiated in RPMI 1640 containing 10% FBS (A5669701, Gibco), 1% penicillin-streptomycin (2587131, Gibco), and 0.02 mg/mL M-CSF (315-02,

PeproTech). After seven days, cell morphology was inspected. Mature BMDMs were harvested, enumerated, and utilized for downstream in vitro or in vivo applications. SPP1 deletion was confirmed by Western blot with anti-SPP1/Osteopontin antibody (6696-RBM3-P1, NeoBiotechnologies).

Flow cytometry analysis

Fresh human tumor and matched adjacent tissues were dissociated using the established protocol. Resulting single cells were incubated with manufacturer-specified concentrations of fluorescent antibodies for 45 min at 4 °C, then washed in PBS. Macrophage staining employed Invitrogen conjugates: CD68-FITC, CD68-PE, IL-6-BV480, IL-10-PE/Cyanine7, TNF- α -BV421, Arg-1-PerCP, PD-L1-APC/Cyanine7, CD163-PE, SPP1-APC, and FABP4-FITC. Phagocytosis was assessed after 20-min incubation with pHrodo Green Dextran (P35368, Invitrogen), followed by staining with CD68-PE/Cyanine7, SPP1-APC, and FABP4-APC. Phagocytic function was quantified via FITC mean fluorescence intensity and positive cell fraction. NK cells and Tregs were labeled using BioLegend reagents: CD3-PerCP, CD56-APC, CD4-PE, and CD25-BV421. Exhausted and cytotoxic T cells were evaluated with BioLegend antibodies against CD3-PerCP, CD4-PE/Cyanine7, CD8-FITC, PD-1-PE, and LAG3-APC.

Xenograft tumors were minced through a 200-mesh screen to create single-cell suspensions. Red blood cells were removed with lysis buffer (130-094-183, Miltenyi). After washing and centrifugation (2000 rpm, 5 min), cells were blocked with CD16/32 and stained for macrophages (CD45-FITC, F4/80-PE, CD11b-PerCP-5.5, CD206-APC), PD1⁺ CD8⁺ T cells (CD3-PE-Cy7, CD4-PE, CD8 α -FITC, CD279-APC), and Tregs (CD4-PE, Foxp3-APC).

BMDMs were polarized for 48 h using 20 ng/mL IL-4 (214-14, PeproTech) + IL-13 (210-13, PeproTech), 20 ng/mL IFN- γ (315-05, PeproTech) + 100 ng/mL LPS (tlrl-pglps, InvivoGen), or CMT64-conditioned medium (1:1 with fresh medium). Cells were stained with CD45-FITC, F4/80-PE, CD11b-PerCP-5.5, CD86-PE-Cy7, CD206-APC, IL-10-FITC, and Arg-1-APC.

Data acquisition was performed on a BD FACS Canto II cytometer and analyzed in FlowJo v10.8.1 (RRID: SCR_008520).

Reverse transcription-quantitative PCR (RT-qPCR)

WT or SPP1^{-/-} BMDMs (1×10^6 cells per well in 6-well plates) were stimulated with 20 ng/mL IL-4 and IL-13 for 48 h. RNA was extracted with TRIzol (15596026CN, Invitrogen), converted to cDNA (RR047A, TaKaRa), and analyzed for immunosuppressive gene expression using TB Green reagent (RR820A, TaKaRa).

β-Galactosidase staining

Senescence in macrophages was measured using the Senescence β-Galactosidase Staining Kit (9860, Cell Signaling Technology) according to the supplied protocol. In brief, 1×10^5 WT or SPP1^{-/-} BMDMs were seeded in 12-well plates, treated with 20 ng/mL IL-4 plus IL-13 for 48 h, fixed, and incubated overnight at 37 °C with staining solution. The fraction of positive (blue) cells was determined by imaging and counting.

In vitro co-culture experiments

CMT64 mouse LUAD cells (RRID: CVCL_2406) were sourced from Fenghui Biotechnology (Hunan, China). For growth assays, polarized (IL-4/IL-13-treated) WT or SPP1^{-/-} BMDMs were placed in Transwell inserts. CMT64 cells were seeded below (1×10^4 for viability, 5×10^4 for proliferation) in 24-well plates. After 1, 3, or 5 days, viability was assessed by CCK-8 assay (C0037, Beyotime) and direct cell counting.

Colony formation was assessed by plating 1×10^3 CMT64 cells per well in 6-well plates with 4×10^5 polarized BMDMs in the upper chamber. After 7 days of co-culture, colonies were fixed, stained, and enumerated. Migration was tested by seeding 1×10^5 polarized BMDMs in the lower chamber and 5×10^4 CMT64 cells in the upper insert in serum-free medium. After 24 h, migrated cells on the underside were fixed, stained, photographed in 20 random fields, and quantified.

In vivo experiments

Luciferase-tagged CMT64 cells were produced by lentiviral transduction (pLV-CMV-LUC, Fenghui Biotechnology, MOI 100) followed by puromycin selection (3 µg/mL). Mice received subcutaneous injections of 3×10^5 CMT64-luc cells either alone or combined with 6×10^5 IL-4/IL-13-polarized WT or SPP1^{-/-} BMDMs into the inguinal area. Tumor volumes were recorded every other day beginning on day 10. On day 25, bioluminescence was quantified after D-Luciferin administration (122799, PerkinElmer) using the Newton FT500 system (Vilber). Animals were humanely euthanized, tumors excised, photographed,

weighed, and dissociated for immune profiling by flow cytometry. All procedures were approved by the Army Medical University Laboratory Animal Welfare and Ethics Committee (AMUWEC20234511).

For CD8 and CD44 blockade experiments, 200 µg anti-CD8α (A2102, RRID: AB_3099521, Selleck) and anti-CD44 (BE0445, RRID: AB_3696168, BioXCell) were injected intraperitoneally one day before cell inoculation and every other day afterward.

Statistical analyses

Analyses and graphing were conducted in SPSS 23.0 (IBM, RRID: SCR_002865) and GraphPad Prism 8.0.1 (RRID: SCR_002798). Two-group comparisons used Student's t-test or Mann-Whitney U test based on variance equality. Multi-group data were analyzed by one-way ANOVA with Tukey or Dunnett's T3 post-hoc tests as appropriate. Categorical variables were assessed by chi-square or Pearson tests. Survival curves were compared using the Kaplan-Meier method. Significance was set at $p < 0.05$.

Results and Discussion

Leukocyte populations undergo substantial shifts across normal lung, GGR, and SR tissues

To characterize cellular changes accompanying mGGN progression, scRNA-seq was performed on 12 matched samples (normal lung, GGR, and SR), generating 122,720 high-quality cells (**Figure 1a**). Unsupervised clustering produced 25 distinct clusters that were classified into seven major lineages—alveolar cells, endothelial cells, epithelial cells, fibroblasts, myeloid cells, T cells, and B cells—using canonical marker genes [23, 24] (**Figures 1b and 1c**). Samples from different tissue regions displayed clear separation in their transcriptional landscapes based on UMI counts (**Figures 1c and 1d**), with characteristic marker genes enriched in each lineage (**Figure 1e**). Proportions of alveolar cells (Chi-square test, $P < 0.001$), endothelial cells, and fibroblasts progressively rose from normal tissue to GGR and further to SR, reflecting extensive tissue remodeling during tumor and microenvironment development. Immune cells (leukocytes) consistently represented the largest compartment in terms of both relative abundance and absolute numbers across all regions, highlighting their likely central contribution to malignant progression in mGGNs. The 96,095 immune cells were subdivided into 22 finer clusters. Among

immune subsets, the macrophage compartment (including alveolar and monocyte-derived macrophages) showed the most dramatic transcriptional reconfiguration as it progressed from normal lung to GGR to SR.

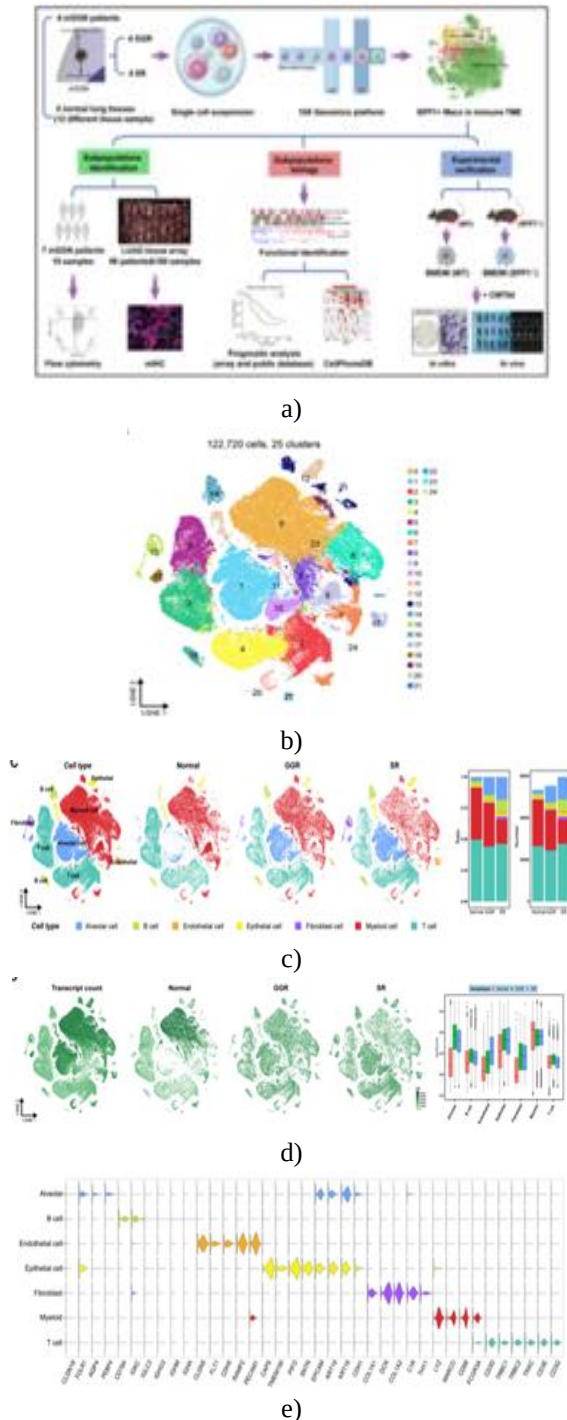


Figure 1. Cell type analysis of paired normal, GGR, and SR tissues based on 122,720 single cells.

(a) Summary diagram illustrating the origins of the tissues examined along with the corresponding validation studies. (b) t-SNE representation displaying 25 color-coded clusters derived from the full set of 122,720 individual cells. (c) Left: t-SNE projection of the seven main cell lineages across normal (n = 4), GGR (n = 4), and SR (n = 4) samples. Right: Bar graph showing the relative distribution of these lineages, with each lineage indicated by a distinct color. (d) Left: t-SNE plot colored by transcript detection levels (ranging from white to green). Right: Boxplot comparing the number of detected transcripts per cell for the seven primary lineages in normal lung, GGR, and SR tissues. (E) Violin plots depicting the expression patterns of lineage-specific marker genes across the seven major cell populations.

Myeloid cell populations drive the establishment of a strongly immunosuppressive tumor microenvironment during mGaN advancement

Given the critical involvement of macrophages in shaping the early pro-tumorigenic niche, we conducted a detailed characterization of myeloid lineage subsets throughout the progression of mGaN. In total, 53,231 myeloid cells from nine initial subsets were resolved into 21 separate clusters. Alveolar macrophages represented the dominant myeloid population and were identified in every tissue sample, whereas monocyte-derived macrophages appeared primarily within the GGR and SR compartments (**Figures 2a and 2b**). As tissues transitioned from normal lung to GGR and then to SR, the relative abundances of alveolar macrophages and monocytes declined sharply. In contrast, the fractions of monocyte-derived macrophages and dendritic cells rose considerably (**Figure 2c**) (Chi-Square test, $P = 0.003$). Interestingly, alveolar macrophages across all stages exhibited strong expression of anti-inflammatory markers (**Figure 2b**). Pathway activity assessment via GSVA showed substantial downregulation of IFN- α/γ signaling, reactive oxygen species generation, and cholesterol homeostasis — functions typically associated with alveolar macrophages [25] — in both GGR- and SR-originating alveolar macrophages (**Figure 2d**) as well as monocyte-derived macrophages (**Figure 2e**). Trajectory reconstruction using pseudotime further uncovered a continuous progression from monocytes into monocyte-derived macrophages, during which genes linked to immune suppression became increasingly expressed (**Figures 2g and 2f**).

Collectively, these observations suggest that both macrophage subsets shift toward a predominantly immunosuppressive phenotype during the early stages of malignant conversion of a lung nodule. While increased levels of IL-1 β and CXCL8 pointed to possible pro-inflammatory activity by granulocytes, the overall proportion of granulocytes was notably reduced in the GGR and SR regions (**Figure 2c**).

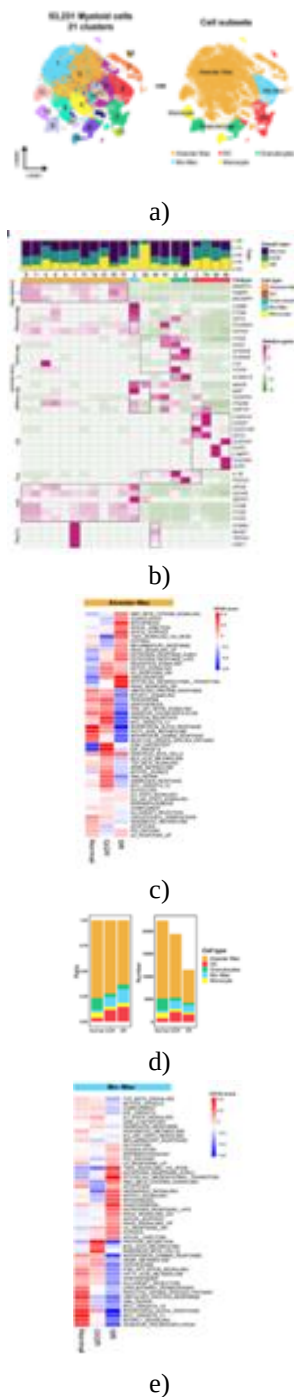


Figure 2. Myeloid cells: dynamic cellular and molecular changes in mGGN.

(a) t-SNE plot of myeloid cells colored by cluster identity (left) and by major population subtypes (right). (b) Heatmap generated with ComplexHeatmap illustrating the proportional distribution of myeloid clusters (top: normal, GGR, and SR) and the z-score-normalized expression levels of selected marker genes across all myeloid populations (bottom; scaled from -3 to 3). (c) Proportional abundances and population breakdowns of different myeloid subsets in normal ($n = 4$), GGR ($n = 4$), and SR ($n = 4$) tissues. (d) GSVA-based pathway activity heatmap in alveolar macrophages across normal, GGR, and SR samples, highlighting differences in signaling pathway enrichment scores. (e) GSVA-based pathway activity heatmap in monocyte-derived macrophages across normal, GGR, and SR samples, showing variations in signaling pathway scores. (f) Monocle 2-generated transcriptional trajectory of monocytes transitioning into monocyte-derived macrophages, colored according to clusters and cell subtypes. (g) Dynamic expression patterns of M2-like macrophage marker genes projected along the pseudotime trajectory derived from the t-SNE plot in panel F.

CD1c $^{+}$ DCs (Langerhans cells, LCs), CD207 $^{+}$ CD1a $^{+}$ LCs, CCR7 $^{+}$ LAMP3 $^{+}$ DCs (activated DCs), CD141 $^{+}$ XCR1 $^{+}$ DCs (cross-presentation DCs), and plasmacytoid dendritic cells (pDCs) constitute the DC population [26] in mGGN. The fraction of CD207 $^{+}$ CD1a $^{+}$ LCs rose substantially in both GGR and SR relative to normal lung, even though overall LC

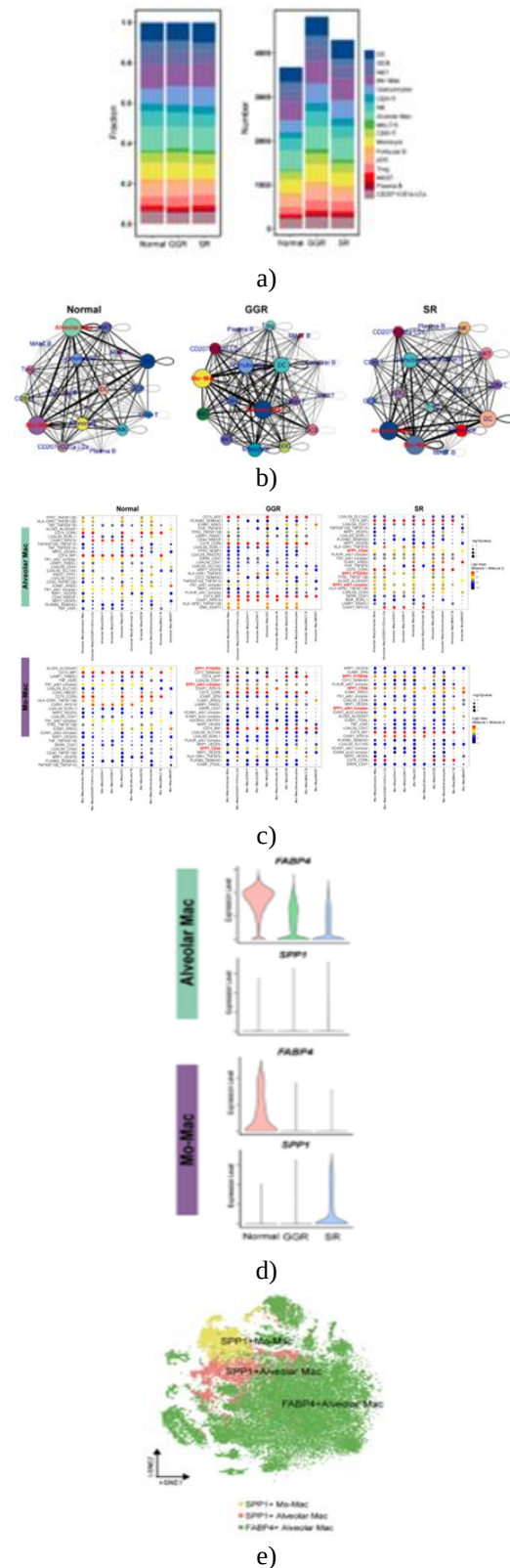
numbers did not change significantly. This shift implies that CD207+CD1a+ LCs may help sustain chronic inflammatory conditions that support pulmonary nodule formation and its subsequent malignant transformation. Except for pDCs, the remaining DC subsets expressed varying levels of co-stimulatory molecules and MHC components. In contrast, pDCs displayed an immunosuppressive profile characterized by upregulated expression of genes from the leukocyte immunoglobulin-like receptor (LILR) family [27].

Immunosuppressive SPP1+ macrophages drive the formation of pro-tumorigenic niches

We subsequently investigated how different macrophage populations (alveolar and monocyte-derived) contribute to building the early pro-tumorigenic microenvironment. CellPhoneDB-based interaction mapping revealed that macrophage subsets consistently served as central hubs in intercellular communication networks across all tissue regions (**Figures 3a and 3b**). Notably, evaluation of ligand-receptor interaction strengths identified three SPP1-associated pairs that were active within both macrophage types during mGGN progression (**Figure 3c**). One of these, the SPP1/PTGER4 axis, has previously been linked to the development of immunosuppressive barriers and tumor-promoting niches in intestinal adenomas [16, 28]. Drawing on earlier work that highlighted FABP4+ macrophages as a major subset in LUAD [16], we specifically tracked SPP1 and FABP4 expression in further analyses.

In both alveolar and monocyte-derived macrophages, FABP4 levels declined while SPP1 expression rose as malignancy advanced in mGGNs (**Figure 3d**). We then partitioned the macrophage populations into SPP1+ and FABP4+ subsets (**Figure 3e**) and tracked their abundance changes with increasing tissue malignancy (**Figure 3f**). Although FABP4+ alveolar macrophages remained the most prevalent overall, their numbers declined markedly during malignant progression. Conversely, SPP1+ monocyte-derived macrophages increased significantly (Chi-Square test, $p = 0.048$). However, their absolute counts stayed relatively modest in solid regions, possibly reflecting infiltration challenges in these denser areas. Independent validation through flow cytometry on fresh surgical mGGN specimens confirmed the reliability of these scRNA-seq observations (**Figure 3g**). Additionally, disease contribution scoring demonstrated that SPP1+ macrophages exerted the greatest influence

among immune cells on the malignant transformation and advancement of mGGNs.



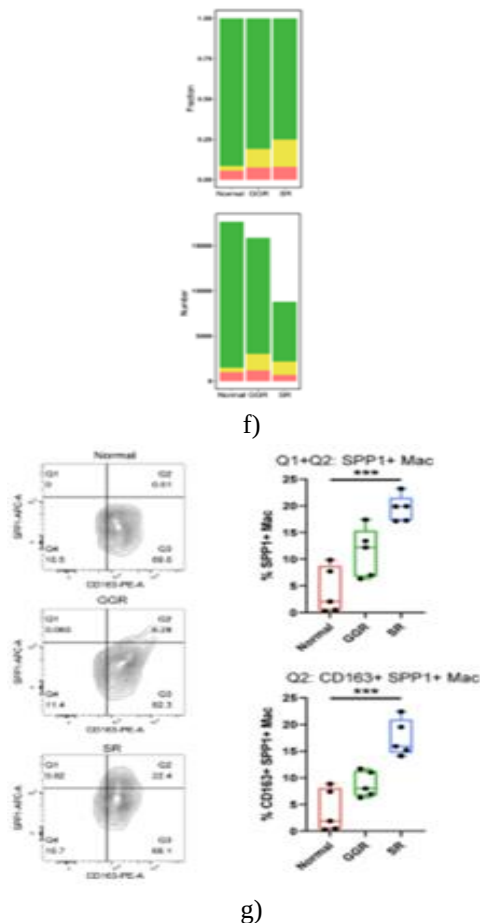


Figure 3. Cell-cell communication between SPP1+ macrophages and other immune populations is markedly strengthened during malignant transformation of mGGNs.

(a) CellPhoneDB analysis showing the quantity of interactions for each immune cell type, presented as a fraction (left) and absolute number (right). (b) Overall predicted interaction network among immune cell subpopulations; line thickness indicates interaction strength and circle size reflects the relative abundance of each subset. (c) Top 25 ligand-receptor pathways identified by CellPhoneDB between macrophages (alveolar and monocyte-derived) and other immune subsets as mGGN malignancy advances. (d) Expression dynamics of FABP4 and SPP1 in alveolar macrophages and monocyte-derived macrophages during mGGN progression. (e) t-SNE visualization of three macrophage subpopulations within the total macrophage pool, each labeled with a distinct color. (f) Quantitative shifts in the three macrophage subpopulations across normal, GGR, and SR tissues ($n = 4$ per group), shown as proportions

(left) and absolute cell numbers (right). (g) Flow cytometry-based quantification of SPP1+ macrophage changes across different mGGN tissue regions. ***, $P < 0.001$.

In addition, we examined the spatial arrangement of SPP1+ macrophages within mGGNs and assessed their clinical prognostic significance in lung cancer. Given the pronounced anti-inflammatory profile observed in macrophages (**Figure 2b**), we conducted multicolor immunohistochemistry using CD68, CD163, and SPP1 markers on mGGN specimens. Compared with matched normal lung tissue, macrophages in mGGN samples showed pronounced spatial reorganization (**Figure 4a**). Notably, SPP1+ macrophages displayed striking heterogeneity in their localization: the majority of SPP1+CD163+ double-positive macrophages accumulated around tumor nests, whereas only sparse SPP1+CD163- macrophages were detected inside the nests. This distribution pattern reinforces the notion that anti-inflammatory SPP1+ macrophages contribute to the establishment of early immunosuppressive barriers.

We extended the same multicolor IHC analysis to a tissue microarray cohort comprising 98 LUAD patients. In advanced LUAD cases, the percentages of both CD68+SPP1+ macrophages and CD68+CD163+SPP1+ macrophages were significantly elevated, although the rise in triple-positive CD68+CD163+SPP1+ cells was more modest (**Figure 4b**). Survival analysis indicated that a higher proportion of CD68+CD163+SPP1+ macrophages was associated with poorer overall survival ($P = 0.013$). A comparable but non-significant trend was observed in stage I LUAD patients (**Figure 4c**). Dual-variable prognostic evaluation using TCGA-LUAD data further revealed that individuals with concurrent high SPP1 expression and elevated M2 macrophage infiltration experienced the least favorable outcomes. However, statistical significance was not reached (**Figure 4d**). Moreover, LUAD tumors with a greater abundance of CD68+CD163+SPP1+ macrophages tended to exhibit higher T, N, and overall TNM stage. Pan-cancer analysis of the TISCH database demonstrated that SPP1 expression is predominantly restricted to monocyte-derived macrophages across multiple human cancer types.

Taken together, these results highlight that anti-inflammatory SPP1+ macrophages are instrumental in building immunosuppressive barriers that accelerate and intensify the malignant progression of mGGNs into lung cancer.

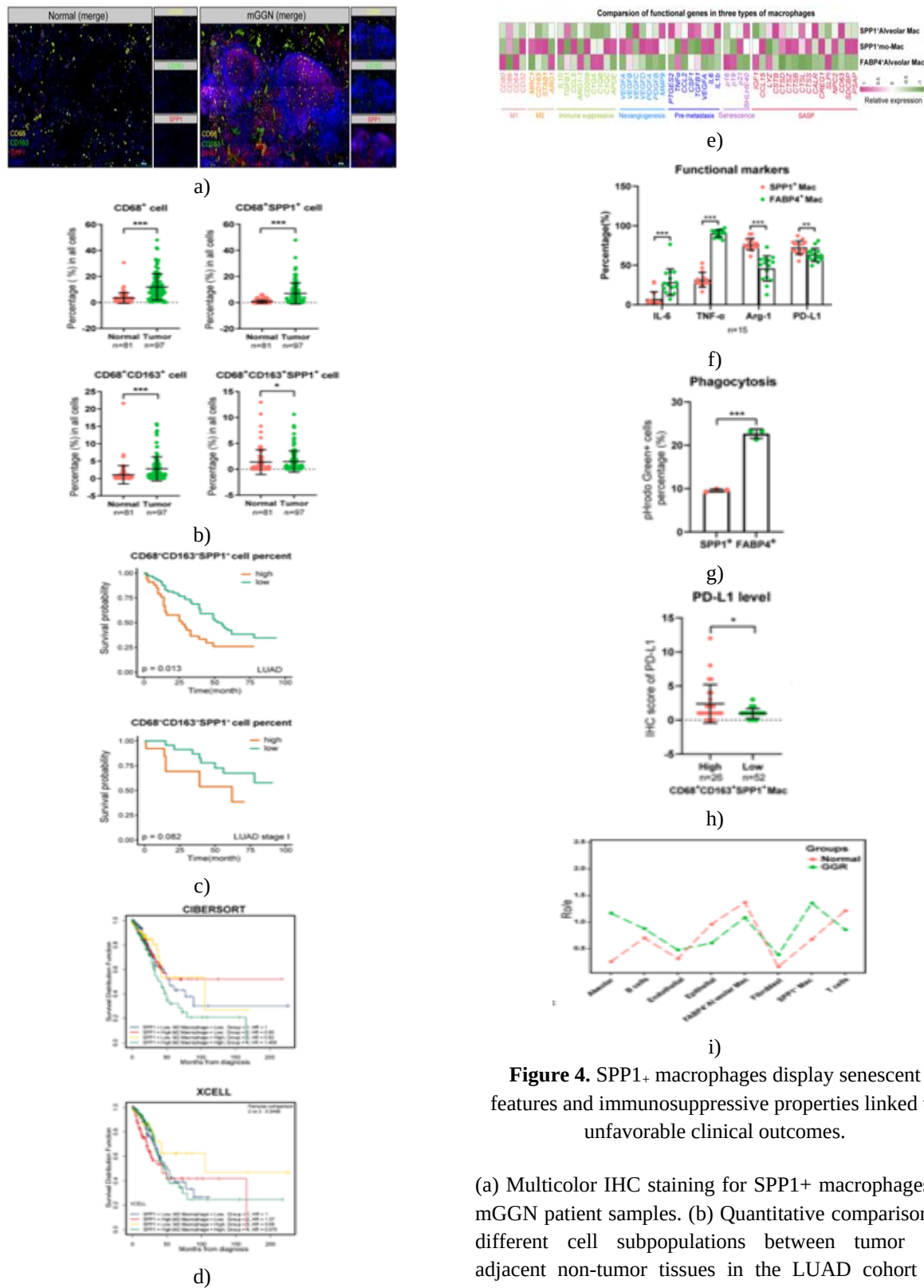


Figure 4. SPP1+ macrophages display senescent features and immunosuppressive properties linked to unfavorable clinical outcomes.

(a) Multicolor IHC staining for SPP1+ macrophages in mGNN patient samples. (b) Quantitative comparison of different cell subpopulations between tumor and adjacent non-tumor tissues in the LUAD cohort via mIHC. (c) Survival analysis of immunosuppressive SPP1+ macrophage infiltration in the full LUAD cohort (high n = 33, low n = 64) and in stage I patients (high n

= 13, low n = 23) from the tissue array. (d) Bivariate prognostic analysis using TCGA-LUAD data with two deconvolution algorithms (CIBERSORT and xCell). CIBERSORT groups: SPP1 low/M2 low (n = 113), SPP1 high/M2 low (n = 106), SPP1 low/M2 high (n = 83), SPP1 high/M2 high (n = 136). xCell groups: SPP1 low/M2 low (n = 110), SPP1 high/M2 low (n = 109), SPP1 low/M2 high (n = 86), SPP1 high/M2 high (n = 133). (e) Distinct molecular expression signatures of the three macrophage subpopulations. (f) Bar graphs depicting differences in immune function markers (IL-6, TNF- α , Arg-1, and PD-L1) between SPP1⁺ and FABP4⁺ macrophages in mixed GGN tissues (n = 15 patients). (g) Bar graphs showing differences in phagocytic ability between SPP1⁺ and FABP4⁺ macrophages in mixed GGN tissues (n = 3). (h) PD-L1 expression levels in relation to high versus low infiltration of CD68⁺CD163⁺SPP1⁺ macrophages in the LUAD cohort. (i) Relative abundance shifts in the three macrophage subsets and other immune populations during transition from normal lung to GGR. *, P < 0.05; ***, P < 0.001. SPP1⁺ macrophages display a senescent phenotype and actively remodel the TME during mGGN malignant progression

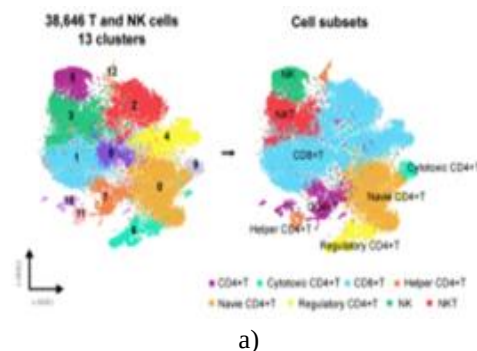
We then compared functional distinctions among the three macrophage populations (FABP4⁺ alveolar macrophages, SPP1⁺ alveolar macrophages, and SPP1⁺ monocyte-derived macrophages). Analysis of differentially expressed functional genes showed that SPP1⁺ macrophages strongly upregulated senescence-related genes and possessed enhanced pro-metastatic and pro-angiogenic potential. SPP1⁺ monocyte-derived macrophages expressed higher levels of M2-associated markers and immunosuppressive genes than the other two subsets, suggesting a dominant role in establishing an immunosuppressive microenvironment (**Figure 4e**). Functional enrichment analysis of genes differentially expressed between FABP4⁺ and SPP1⁺ alveolar macrophages revealed stronger activation of immune regulatory pathways in SPP1⁺ cells, consistent with earlier observations during mGGN progression. GO and KEGG analyses confirmed elevated enrichment of immune response modulation pathways in SPP1⁺ alveolar macrophages. GSVA analysis further demonstrated higher activity scores for immunosuppressive pathways in SPP1⁺ macrophages compared with FABP4⁺ macrophages. These data imply that SPP1⁺ alveolar macrophages exert a particularly

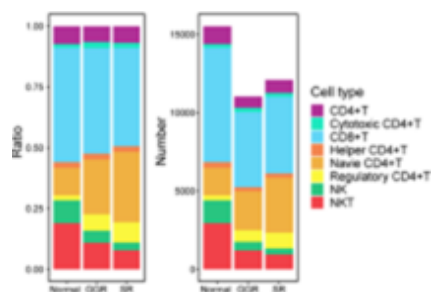
strong negative regulatory effect on immune responses [29].

Flow cytometric validation in mixed GGN clinical specimens corroborated these molecular findings. SPP1⁺ macrophages contained a significantly higher fraction of Arg-1⁺/PD-L1⁺ (immunosuppressive) cells and a lower fraction of TNF- α ⁺/IL-6⁺ (immunoactivating) cells compared with FABP4⁺ macrophages (**Figure 4f**). A modest, non-significant increase in IL-10⁺ cells was also noted in SPP1⁺ macrophages. Additionally, SPP1⁺ macrophages displayed reduced phagocytic function, evidenced by lower percentages of pHrodo Green-positive cells and decreased mean fluorescence intensity (**Figure 4g**).

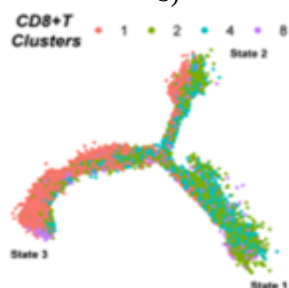
Interestingly, PD-L1 protein abundance in LUAD tumors positively correlated with the density of infiltrating CD68⁺CD163⁺SPP1⁺ macrophages (**Figure 4h**). Ro/e analysis further revealed that SPP1⁺ macrophage distribution was inversely associated with T cell infiltration during the transition from normal lung to GGR — the initial phase of malignant evolution (**Figure 4i**). Collectively, these observations establish SPP1⁺ macrophages as major drivers of TME remodeling that favor malignant progression in mGGNs. Infiltrating T cells and natural killer (NK) cells progressively decline and acquire an exhausted phenotype

T cells and NK lineage cells were partitioned into eight subpopulations as previously defined (**Figure 5a**). The proportions of CD8⁺ T cells and NK/NKT cells declined sharply from normal tissue through GGR to SR. In contrast, naïve CD4⁺ T cells and regulatory CD4⁺ T cells showed notable increases in GGR and SR compared with normal lung (Chi-Square test, P = 0.006) (**Figure 5b**).

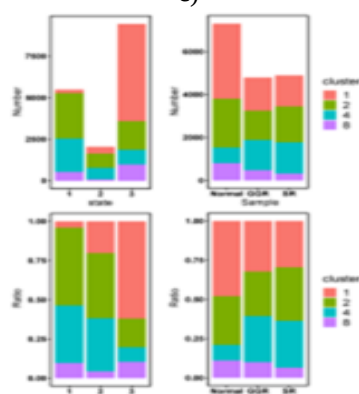




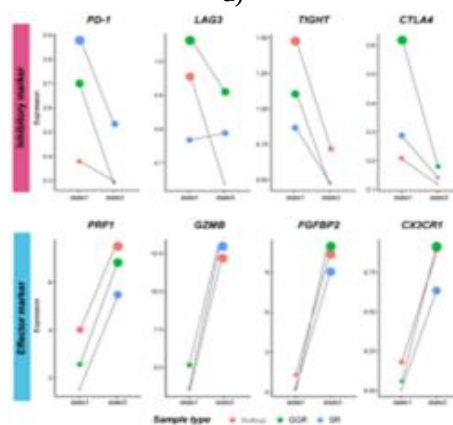
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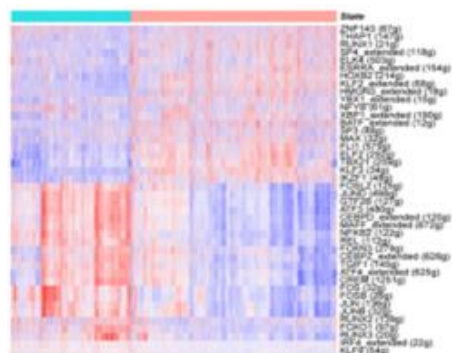
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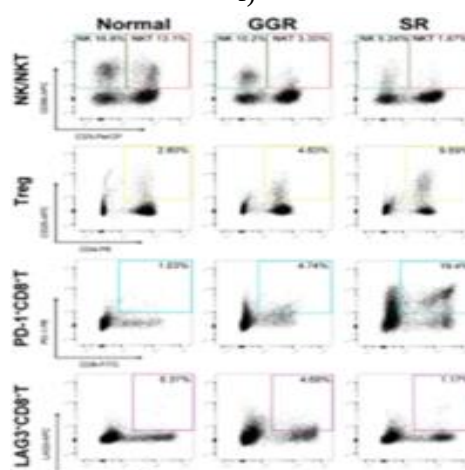
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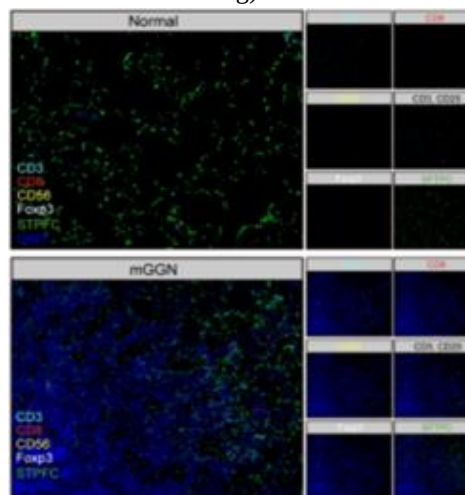
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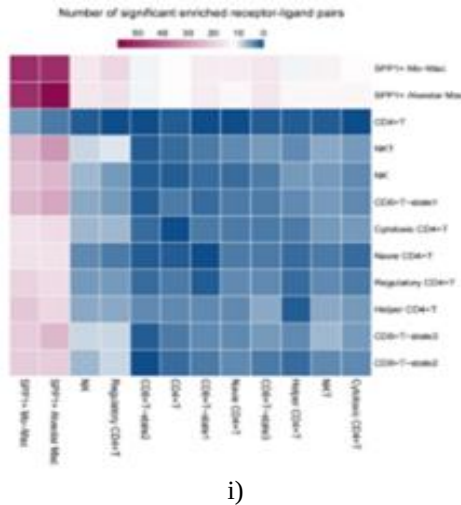


Figure 5. Dynamic cellular and molecular alterations of T and NK cells during mGaN progression.

(a) t-SNE visualization of T and NK lineage cells, with coloring by individual clusters (left) and by specific cell subtypes (right). (b) Proportions and absolute counts of various T and NK cell subtypes across normal, GGR, and SR tissues (n = 4 samples per group). (c) Pseudotime developmental trajectory of the four CD8⁺ T cell subclusters constructed with Monocle 2, colored according to subcluster identity. (d) Changes in proportions and cell numbers for the four CD8⁺ T cell subsets across the three tissue conditions and states. (e) Bubble plot highlighting shifts in expression of inhibitory versus effector molecules across two functional states of CD8⁺ T cells. Colors indicate tissue origin (normal, GGR, or SR). (f) Heatmap of regulon activity scores (AUC) for key transcription factors determined by SCENIC. (g) Flow cytometry examples of alveolar type II cells, NK cells, and selected T cell populations (including Tregs and exhausted CD8⁺ T cells) from GGR, SR, and matched normal lung samples (n = 4). (h) Multicolor IHC images illustrating spatial localization of total T cells, CD8⁺ T cells, NK cells, NKT cells, and regulatory T cells in the three tissue compartments. Solid regions (SR) within mGGN exhibit lower SFTPC levels and higher cellular density. (i) Heatmap (log10 scale) summarizing the number of significant ligand-receptor interactions between SPP1+ macrophages and various T/NK cell subsets.

To gain deeper insight into CD8⁺ T cell heterogeneity across normal lung, GGR, and SR tissues, we reconstructed functional trajectories for the four identified CD8⁺ T cell clusters. Infiltrating CD8⁺ T cells

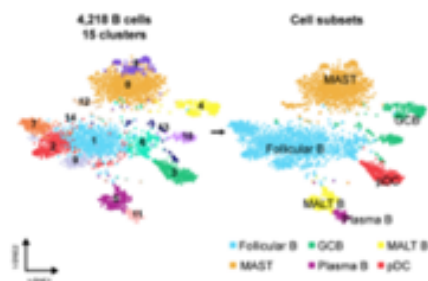
exhibited three distinct functional states defined through differential gene expression (**Figure 5c**). Clusters 1 and 8 were mainly derived from normal tissue and aligned with State 3, whereas clusters 2 and 4 were enriched in GGR and SR samples and corresponded primarily to State 1 (**Figure 5d**).

Pathway activity comparison (GSVA) between State 3 and State 1 demonstrated strong enrichment of cytotoxic programs — such as antigen recognition, TCR signaling and effector molecule production — in State 3. State 1 cells (clusters 2 and 4) showed elevated exhaustion markers including LAG3 and PDCD1 (PD-1), while State 3 cells (clusters 1 and 8) expressed higher levels of granzymes and IFN- γ . This indicates that State 1 CD8⁺ T cells are undergoing a transitional process toward exhaustion, supported by GZMK expression [30]. CD8⁺ T cells in State 1 from GGR and SR tissues upregulated inhibitory and transitional molecules while downregulating effector genes relative to normal tissue. Overall, cytotoxic CD8⁺ T cell populations progressively diminished and converted into exhausted phenotypes as mGGN malignancy advanced.

Notably, State 1 CD8⁺ T cells in SR regions displayed upregulation of CD28 and PD-1, supporting the potential efficacy of PD-1 checkpoint inhibition in solid pulmonary nodules [31, 32]. In GGR, these cells instead showed prominent LAG3 and CTLA4 expression (**Figure 5e**). SCENIC-based regulatory network analysis revealed elevated activity of transcription factors involved in effector function maintenance (KLF2, KLF3, BATF, TBX21/T-bet) in State 3. In contrast, regulons linked to naïve/memory phenotypes (FOXO1, TCF7) and exhaustion (IRF4, EOMES) [33] were more active in State 1. Activation of AP-1 components (FOS, JUN), NFkB2, and RUNX3 in State 1 likely sustains limited IL-2 production, particularly in cluster 4 (**Figure 5f**). Heightened proliferation gene signatures further suggest that State 1 cells represent an early dysfunctional phase originating from formerly cytotoxic populations [30, 34]. NK cells (cluster 5) and NKT cells (cluster 3) retained strong IFN- γ and granzyme secretion potential, implying an important role in restraining early malignant growth in LUAD. Nevertheless, STEM analysis indicated gradual loss of effector capabilities in GGR and SR. Heat shock protein expression [35, 36], critical for NK/NKT activation and maintenance, declined progressively across the tissue stages, while the immunosuppressive factor METRN1 increased, consistent with functional decline. Flow cytometry and multicolor IHC validation

confirmed decreasing trends in CD8⁺ T cells and NK/NKT cells, alongside rising Treg infiltration during mGGN progression (**Figures 5g and 5h**). Intercellular communication mapping highlighted robust interactions between SPP1⁺ macrophages and T/NK subsets (**Figure 5i**). B cells accumulate within mGGN without developing typical tertiary lymphoid structures.

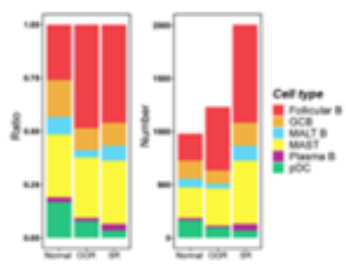
B cells and associated tertiary lymphoid structures are recognized as important players in both tumor formation and immunotherapy responses [37, 38]. From the remaining 4,218 leukocytes, we identified 15 clusters that grouped into six B cell-related subpopulations (**Figures 6a and 6b**). Total B cell abundance rose substantially in GGR and SR compared to normal lung tissue, consistent with previous findings linking higher intratumoral B cell numbers to earlier lung cancer stages [38]. As nodule composition evolved, follicular B cells and plasma cells increased markedly, while germinal center B cell populations declined (**Figure 6c**) (Chi-Square test, $P = 0.009$).



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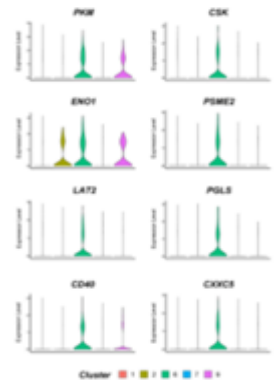
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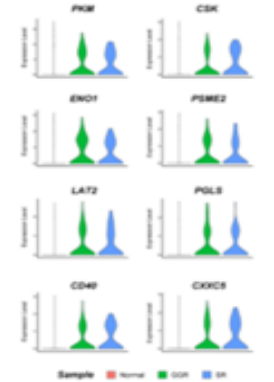
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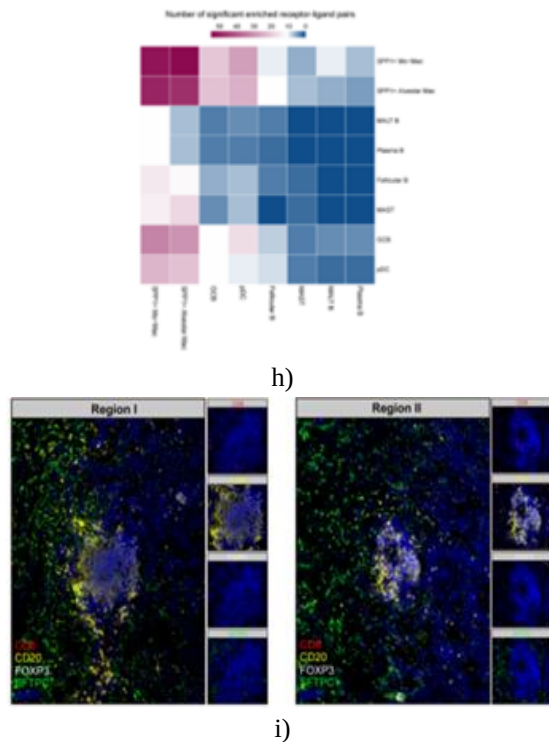


Figure 6. Dynamic cellular and molecular alterations of B cells and pDCs during mGGN progression.

(a) t-SNE visualization of B cells and pDCs colored according to cluster membership (left) and subtype classification (right). (b) ComplexHeatmap displaying proportional distributions and marker gene profiles for B cells and pDCs. Upper section: relative abundance of each cluster in normal, GGR, and SR samples. Lower section: z-score normalized marker gene expression levels across cell types (range -3 to 3). (c) Proportions and population distributions of B cell and pDC subtypes in normal lung, GGR, and SR tissues ($n = 4$ per group). (d) GSVA pathway activity heatmap for follicular B cells across normal, GGR, and SR, illustrating variations in signaling pathway enrichment scores among cell populations. (e) Violin plots of genes showing enriched expression specifically in cluster 6 of follicular B cells. (f) Violin plots comparing expression of cluster 6 signature genes in follicular B cells from GGR and SR relative to normal tissue. (g) GSVA pathway activity heatmap for germinal center B cells in normal, GGR, and SR samples, showing differences in pathway scores. (h) Heatmap of significant ligand-receptor interaction counts between SPP1⁺ macrophages and B cell/pDC subsets. The color scale from dark blue to dark magenta reflects increasing interaction strength. (i) Multicolor IHC images depicting localization of CD20⁺ B cells,

CD8⁺ T cells, Treg cells, and ATII cells across the three tissue compartments. SR regions in mGGN are identified by lower SFTPC expression and elevated cell density (white dotted lines mark potential tertiary lymphoid structures).

GSVA analysis demonstrated that follicular B (FB) cells from GGR and SR exhibited diminished activity scores in pathways governing cell proliferation and differentiation (MYC, mTOR, IL-2, and IL-6 signaling) as well as inflammatory responses (IFN and complement cascades) (**Figure 6d**). This points to functional exhaustion of FB cells even as their numbers expand. Remarkably, cluster 6 (C6), which predominantly originated from normal and GGR tissues, maintained robust antigen presentation and immune stimulatory functions despite the overall anergic trend in the FB compartment. Signature genes of C6 were strongly associated with immune activation, including PKM, CSK, ENO1, PSME2, LAT2, PGLS, CD40, and CXXC5 (**Figure 6e**). These transcripts were upregulated in C6 cells from GGR and SR compared with normal samples (**Figure 6f**). In TCGA LUAD Stage I data, the C6 gene signature from follicular B cells correlated with better overall survival ($P = 0.019$).

Consistent with findings in follicular B cells, germinal center B (GCB) cells in SR showed reduced GSVA scores for proliferation and differentiation pathways, while those in GGR resembled normal tissue and may exert anti-tumor activity during initial tumor development (**Figure 6g**). Clusters 14 and 13, largely from GGR and SR and located in the light zone, showed low MKI67, suggesting a gradual decline in proliferative potential in cancerous environments. Unlike prior observations [23], pDC numbers steadily decreased as tissues transformed (**Figure 6c**) (Chi-Square test, $P = 0.009$). Furthermore, pDCs produced very little type I interferon, potentially impairing immune responses in GGR and SR [39].

Intercellular communication mapping indicated intimate links between SPP1⁺ macrophages and both GCBs and pDCs (**Figure 6h**). Multicolor IHC also showed that CD8⁺ T cells and other effector immune populations rarely infiltrated B cell-rich zones, suggesting that conventional tertiary lymphoid structures do not develop in mGGNs (**Figure 6i**).

SPP1⁺ macrophages engage with immune cells and modify their phenotypes during mGGN malignant progression

To assess the influence of SPP1⁺ macrophages on surrounding immune populations in mGGN, we performed a systematic crosstalk analysis across all immune cells using counts of significantly enriched ligand-receptor pairs. Interaction landscapes differed markedly among normal, GGR, and SR tissues, revealing a progressive intensification of communications orchestrated by SPP1⁺ macrophages (**Figure 7a**). These dynamic shifts, particularly with anti-tumor effector subsets (such as CD8⁺ T cells in State 3 and follicular B cells) and immunosuppressive populations (pDCs, Tregs, and CD8⁺ T cells in State 1), highlight the pivotal contribution of SPP1⁺ macrophages to creating early immunosuppressive barriers and promoting a tumor-supportive niche.

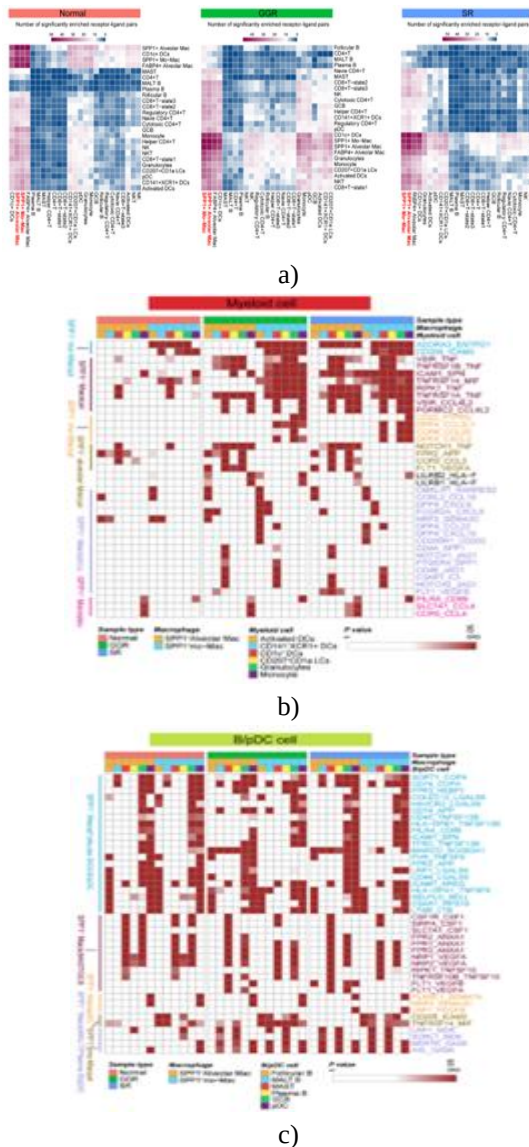


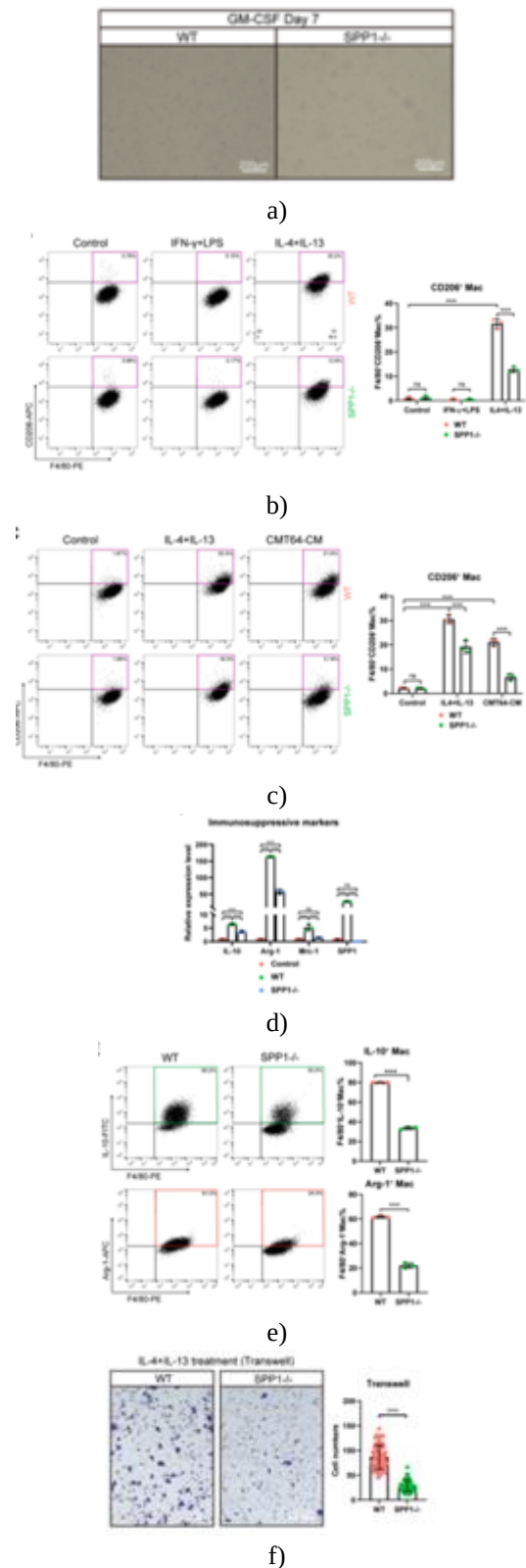
Figure 7. Significant ligand-receptor (L/R) pairs mediating targeted cell-cell communications between SPP1⁺ macrophages and other immune populations.

A Heatmap illustrating the number of significant interactions (Log10 scale) involving SPP1⁺ and FABP4⁺ macrophages with additional immune cell types in (left) normal lung, (middle) GGR, and (right) SR regions. Heatmaps displaying P-values for notable L/R pairs driving specific intercellular communications between B SPP1⁺ macrophages and myeloid cells; C SPP1⁺ macrophages and T/NK cells; D SPP1⁺ macrophages and B cells/pDCs during mGGN progression. Additional examination indicated that SPP1⁺ alveolar macrophages (AM) and monocyte-derived macrophages (Mo-Mac) exerted the most potent influence on other myeloid, T/NK, and B/pDC populations throughout mGGN advancement. As shown in **Figures 7b-7d**, the two SPP1⁺ macrophage subsets displayed distinct interaction profiles with myeloid and T/NK cells, while sharing comparable patterns with B/pDC cells. Notably, the CD209_ICAM3 interaction was detected exclusively in SPP1⁺ Mo-Mac communications with nearly every immune subset, and absent from SPP1⁺ AM interactions. Within the SR area, SPP1⁺ Mo-Macs engaged with monocytes and T/NK cells through CD22_PTPRC. Key signals such as NOTCH1_TNF, CCR5, and FPR2 dominated contacts between SPP1⁺ AM and both myeloid and T/NK cells. In particular, SPP1⁺ AM facilitated monocyte infiltration into mGGN lesions and their subsequent differentiation into Mo-Macs via CCR5, SLC7A1, and PILRA pathways. Collectively, these observations highlight SPP1⁺ macrophages as central regulators that recruit immune cells and drive phenotypic shifts, thereby supporting the onset and advancement of malignant ground-glass nodules.

SPP1 knockout restricts BMDM polarization toward an immunosuppressive, tumor-promoting state

To validate the tumor-promoting activity of SPP1⁺ macrophages in malignant GGN development, bone marrow-derived macrophages (BMDMs) were obtained from wild-type or SPP1-knockout mice, with SPP1 depletion verified via western blotting. Morphologically, SPP1 deletion had no impact on BMDM appearance or yield seven days after M-CSF stimulation (**Figure 8a**). Nonetheless, loss of SPP1 markedly curtailed the immunosuppressive polarization of BMDMs triggered by IL-4 and IL-13 (**Figure 8b**), while leaving IFN- γ and LPS-driven activation unaltered. Moreover, SPP1 deficiency blocked CMT64-conditioned medium-induced immunosuppressive polarization of BMDMs (**Figure 8c**). RT-qPCR and flow cytometry analyses corroborated these observations: typical immunosuppressive markers such as IL-10, Arg-1, and Mrc-1 rose in BMDMs following IL-4/IL-13 exposure, yet this upregulation was substantially attenuated in the absence of SPP1 (**Figures 8d and 8e**). Transwell migration assays further revealed that SPP1 knockout profoundly reduced IL-4/IL-13-stimulated BMDM motility (**Figure 8f**). Prior studies have linked macrophage senescence to immunosuppressive activity and tumor enhancement, with SPP1 identified as a senescence marker in macrophages [40]. Our data likewise imply that SPP1⁺ macrophages within mixed pulmonary nodules display senescent features and contribute to nodule malignancy (**Figures 3 and 4**). We subsequently assessed how SPP1 knockout influenced BMDM senescence. As anticipated, SPP1 deletion led to a notable decline in the proportion of β -Gal-positive BMDMs following IL-4 and IL-13 treatment (**Figure 8g**). Integrating these results, we propose that macrophages lose their tumor-supportive capacity upon SPP1 knockout. To test this directly, we evaluated the impact of SPP1^{-/-} BMDMs on the malignant traits of mouse lung adenocarcinoma CMT64 cells (**Figure 8h**). Following IL-4/IL-13-induced immunosuppressive polarization, wild-type BMDMs enhanced CMT64 cell viability and proliferation, whereas SPP1^{-/-} BMDMs failed to do so (**Figure 8i**). Likewise, IL-4/IL-13-treated wild-type BMDMs substantially increased CMT64 colony formation (**Figure 8j**) and migratory ability (**Figure 8k**), effects that were eliminated by SPP1 knockout. Together, these data establish that SPP1 is essential for the immunosuppressive polarization of

BMDMs and that its absence abolishes BMDMs' ability to drive proliferation and migration in CMT64 cells.



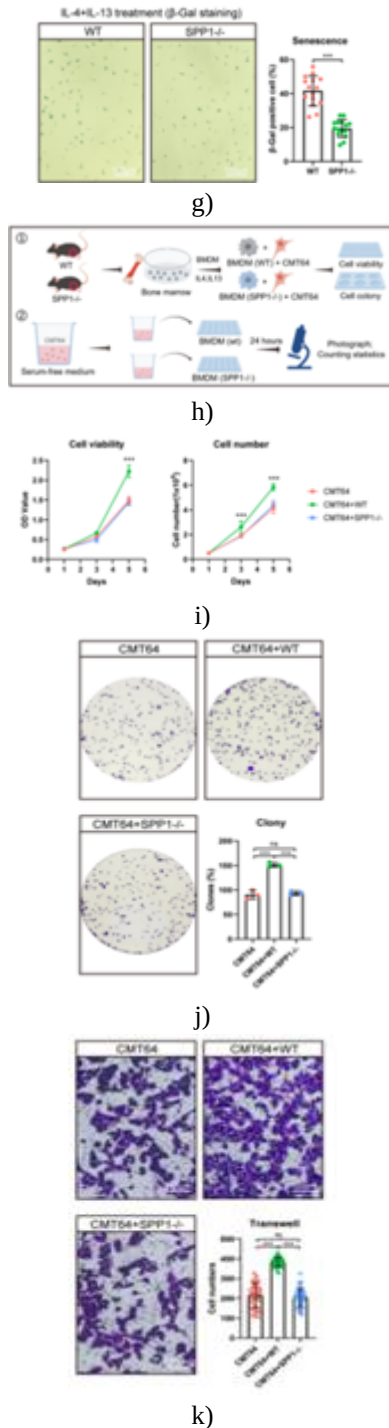


Figure 8. SPP1 deletion restrains BMDM polarization into an immunosuppressive and tumor-promoting state.

(a) Bone marrow-derived macrophages were cultured and imaged. Scale bar = 200 μ m. (b-c) Flow cytometry analysis of CD206+ macrophage percentages under various treatment conditions. Representative flow plots

(left) and quantification of cell proportions (right), $n = 3$. (d-e) Following IL-4 and IL-13 stimulation, (D) mRNA expression of immunosuppressive markers (IL-10, Arg-1, Mrc-1, and SPP1) was measured by RT-qPCR. Histograms display relative expression levels ($n = 3$); (e) percentages of IL-10+ and Arg-1+ macrophages were assessed by flow cytometry after IL-4/IL-13 treatment. Representative flow plots (left) and quantification (right), $n = 3$. (f) Representative transwell images of BMDMs 24 h post IL-4/IL-13 treatment (left). Histogram shows the number of migrated BMDMs (right), $n = 60$. Scale bar = 100 μ m. (g) Representative β -Galactosidase staining images of BMDMs. Positive cells were quantified. Scale bar = 100 μ m (left). Histogram displays the percentage of β -Gal-positive BMDMs, $n = 3$ (right). (h) Schematic overview of the in vitro experimental design. (i) Line graph depicting CMT64 cell viability and proliferation at days 1, 3, and 5 after co-culture with IL-4/IL-13-polarized WT or SPP1-/- BMDMs. CMT64 cells alone served as control, $n = 3$. (J) Representative images of CMT64 colonies on day 7 following co-culture with IL-4/IL-13-stimulated WT or SPP1-/- BMDMs. Histogram shows relative colony percentages (lower right), $n = 3$. (K) Representative transwell images 24 h after co-culture with IL-4/IL-13-stimulated WT or SPP1-/- BMDMs. Histogram quantifies migrated cells (lower right), $n = 60$. Scale bar = 100 μ m. ns, not significant; *, $P < 0.05$; ***, $P < 0.001$. Data pooled from three independent replicates.

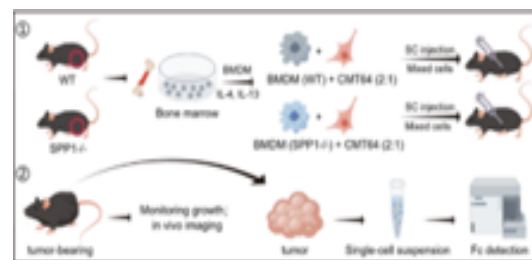
SPP1-/- BMDMs counteract the immunosuppressive tumor microenvironment and restrain CMT64 subcutaneous tumor progression in vivo

Macrophages are a major component of the tumor microenvironment (TME) and actively modulate immune cell function within it [9]. To confirm the influence of SPP1-expressing macrophages on the TME during early-stage lung adenocarcinoma, an in vivo model was established by co-injecting WT or SPP1-/- BMDMs with CMT64-luc cells (2:1 ratio) into the inguinal flank of mice (**Figure 9a**). On day 25 post-injection, bioluminescence imaging showed markedly elevated mean fluorescence intensity in tumors from the CMT64 + WT BMDM group compared to CMT64 alone. In contrast, the CMT64 + SPP1-/- BMDM group exhibited significantly lower fluorescence intensity than the other groups (**Figures 9b and 9c**). Tumor growth curves and final tumor weights further demonstrated that WT BMDMs strongly enhanced CMT64 proliferation in

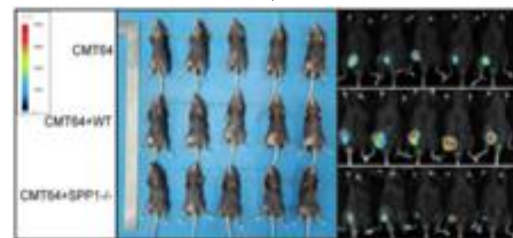
vivo, while SPP1^{-/-} BMDMs suppressed tumor expansion (**Figures 9d and 9f**). Given that SPP1 knockout substantially limits cytokine- or tumor-conditioned medium-driven immunosuppressive polarization of BMDMs (**Figures 8b and 8c**), we hypothesized that the TME in SPP1^{-/-} BMDM co-injected tumors would exhibit reduced immunosuppression compared to WT BMDM co-injections. Flow cytometric profiling of tumor-infiltrating immune cells (**Figures 9g and 9h**) revealed increased frequencies of PD-1⁺CD8⁺ T cells and regulatory T cells (Tregs) in WT BMDM co-xenografts. In contrast, these populations were significantly diminished in SPP1^{-/-} BMDM co-xenografts compared to the other groups. Additionally, the proportion of CD206⁺ immunosuppressive macrophages within the tumors on day 25 was notably lower in the SPP1^{-/-} BMDM co-injection group (**Figure 9i**). Analysis of pro-inflammatory (IL-1 β /2/6, IFN- γ , TNF- α , MCP-1, MIP-1 α) and anti-inflammatory (IL-4/10, TGF- β) cytokines at mRNA and protein levels in tumor tissues showed pronounced upregulation of pro-inflammatory factors in the SPP1-knockout setting compared to WT BMDM or CMT64-only groups. These results suggest that SPP1^{-/-} BMDMs fail to establish an immunosuppressive milieu; instead, they foster anti-tumor immunity by elevating pro-inflammatory mediators in the TME.

To eliminate potential confounding effects from endogenous macrophages on SPP1⁺ macrophage tumor promotion, mice were pre-treated with clodronate liposomes (CL) to deplete resident macrophages before tumor cell inoculation. First, RAW264.7 cells overexpressing SPP1 (OE-spp1) were generated and confirmed to display heightened sensitivity to IL-4/IL-13-induced immunosuppressive polarization. As anticipated, after CL-mediated depletion, OE-spp1 RAW264.7 cells markedly accelerated early CMT64 tumor growth in vivo. Upon CD8⁺ T cell depletion using a CD8 α -blocking antibody, the tumor-suppressive effect observed in the SPP1^{-/-} group was completely reversed, indicating that the anti-tumor activity of SPP1^{-/-} macrophages relies on CD8⁺ T cells (**Figures 9j and 9m**). In vitro co-culture assays verified that SPP1⁺ macrophages strongly inhibited CD8⁺ T cell proliferation and IFN- γ production, whereas SPP1^{-/-} macrophages lost this suppressive ability. To determine whether SPP1⁺ macrophage regulation of CD8⁺ T cells occurs through the SPP1/CD44 pathway, CD44 antagonism experiments were performed in vivo. Tumor growth curves showed

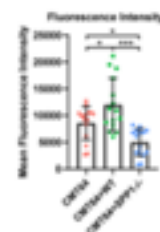
that the tumor-promoting effect of WT BMDMs on CMT64 cells was abrogated by CD44 blockade, resulting in significantly reduced tumor growth rates and weights compared to isotype controls (**Figures 9n and 9q**). Additionally, the SPP1-PTGER4 interaction was examined using the specific inhibitor E7046. In vitro macrophage-CD8⁺ T cell co-cultures demonstrated that E7046 mitigated the inhibitory effect of IL-4/IL-13-stimulated WT macrophages on CD8⁺ T cells, restoring both proliferative capacity (CFSE dilution) and IFN- γ secretion. Overall, these findings establish that SPP1 knockout in BMDMs suppresses CMT64 tumor development in a CD8⁺ T cell-dependent manner, with SPP1⁺ macrophages impairing CD8⁺ T cell function primarily through SPP1/CD44 and SPP1/PTGER4 signaling axes.



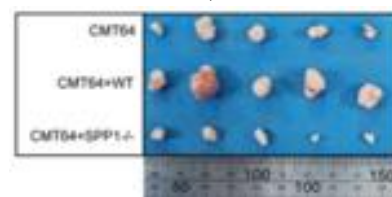
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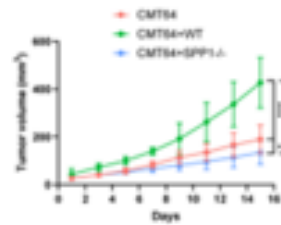
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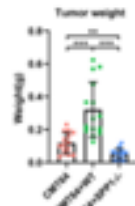
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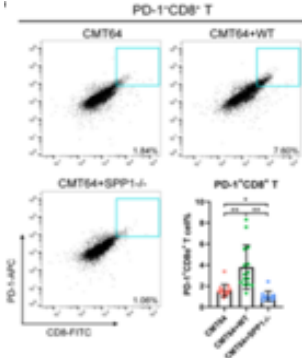
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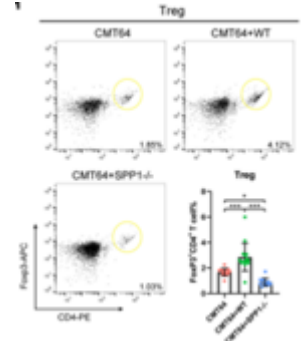
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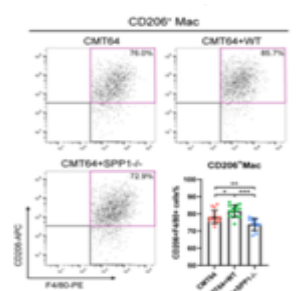
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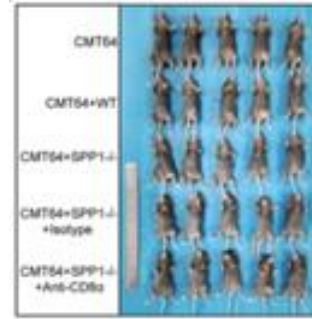
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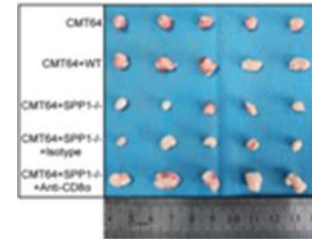
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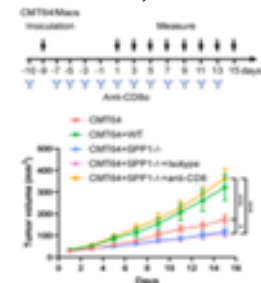
i)



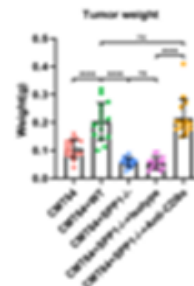
j)



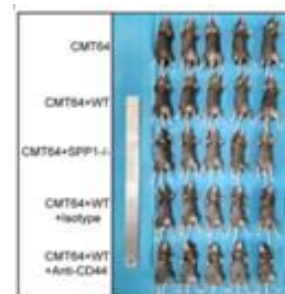
k)



l)



m)



n)

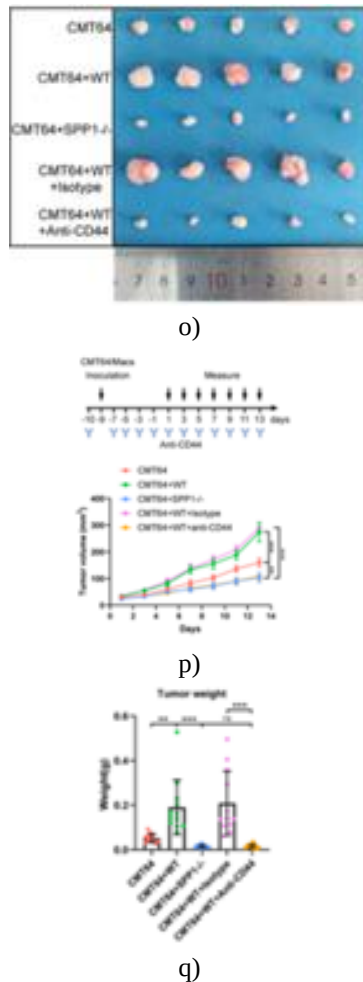


Figure 9. SPP1^{-/-} BMDMs counteract the immunosuppressive tumor microenvironment and inhibit CMT64 xenograft tumor growth in vivo.

(a) Schematic illustration of the in vivo experimental workflow. (b) Representative photographs of tumor-bearing mice (left) and bioluminescence imaging (right). (c) Quantification of mean fluorescence intensity in xenograft tumors from CMT64-luc, CMT64-luc + WT BMDMs, and CMT64-luc + SPP1^{-/-} BMDMs groups (n = 15). (d) Representative images of excised xenograft tumors harvested 25 days after inoculation (n = 5). (e) Tumor growth curves for CMT64-luc, CMT64-luc + WT BMDM, and CMT64-luc + SPP1^{-/-} BMDM groups measured every other day (n = 15). (f) Histogram showing tumor weights from CMT64-luc, CMT64-luc + WT BMDM, and CMT64-luc + SPP1^{-/-} BMDM groups (n = 15). (g-i) Flow cytometry analysis of PD-1⁺CD8⁺ T cells (g), Tregs (h), and CD206⁺ macrophages (i) in xenograft tumors from CMT64-luc (n = 15), CMT64-luc + WT BMDM (n = 15), and CMT64-luc + SPP1^{-/-}

BMDM (n = 12) groups. Histograms display differences in cell percentages (lower right). (j) Representative images of tumor-bearing mice in the anti-CD8α treatment experiments. (k) Representative images of excised xenograft tumors harvested 25 days post-inoculation (n = 5). (l) Schematic of the experimental design (top); tumor growth curves for CMT64, CMT64 + WT, CMT64 + SPP1^{-/-}, CMT64 + SPP1^{-/-} + isotype, and CMT64 + SPP1^{-/-} + anti-CD8α groups (n = 15) (bottom). (m) Histogram of tumor weights from CMT64, CMT64 + WT, CMT64 + SPP1^{-/-}, CMT64 + SPP1^{-/-} + isotype, and CMT64 + SPP1^{-/-} + anti-CD8α groups (n = 15). (n) Representative images of tumor-bearing mice in the anti-CD44 treatment experiments. (o) Representative images of excised xenograft tumors harvested 23 days post-inoculation (n = 5). (p) Schematic of the experimental design (top); tumor growth curves for CMT64, CMT64 + WT, CMT64 + SPP1^{-/-}, CMT64 + WT + isotype, and CMT64 + WT + anti-CD44 groups (n = 12) (bottom). (q) Histogram of tumor weights from CMT64, CMT64 + WT, CMT64 + SPP1^{-/-}, CMT64 + WT + isotype, and CMT64 + WT + anti-CD44 groups (n = 12). ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001. Data pooled from three independent replicates.

Data from the National Lung Screening Trial (NLST) and Chinese cohorts indicate that ground-glass nodules (GGN) represent 84.87% of pulmonary nodules ultimately confirmed as early-stage lung cancer. Early lung cancer carries a 5-year survival rate above 85%, and appropriate early intervention can lower cancer-specific mortality by 20% [41-43]. Elucidating the molecular drivers of malignant progression in pure GGN (pGGN) and developing targeted strategies are therefore critical for improving diagnosis and treatment of patients with pulmonary nodules.

Unlike earlier investigations of pulmonary nodules [44], the present study specifically examined the contribution of highly adaptable tumor-associated macrophages (TAMs) to the establishment of pro-tumorigenic microenvironments during pGGN malignant transformation, with emphasis on identifying pivotal macrophage subsets. In advanced solid lung cancers, extensive molecular communication between tumor cells and TAMs is known to drive tumor expansion and invasion [45-47]. Reprogramming TAMs has demonstrated considerable therapeutic promise in lung cancer [48, 49] and has yielded near-curative outcomes in preclinical models [50]. Nevertheless, research on key

macrophage populations during pGGN progression remains limited, and insights derived from solid tumors may not fully translate to patients harboring pulmonary nodules.

Recent reports have highlighted the significant involvement of SPP1⁺ macrophage populations in cancer advancement [17, 51]. In hepatocellular carcinoma, SPP1⁺ macrophages create an immunosuppressive barrier at the tumor periphery, restricting immune cell penetration into the tumor core [52]. Our study extended this by investigating SPP1⁺ macrophages in pro-tumorigenic niche formation within mixed GGN (mGGN). Consistent with prior observations, multiplex immunohistochemistry revealed that anti-inflammatory SPP1⁺ macrophages localize around cancer nests. Our findings also showed that SPP1⁺ macrophages display senescent features, aligning with reports that senescent alveolar macrophages accumulate early during lung neoplasia in both murine models and human tissues, where they foster tumorigenesis by modulating anti-tumor T cell responses [40, 53]. SPP1⁺ macrophages have additionally been implicated as tumor-promoting cells in smoking-associated lung cancer [54]. These observations support the idea that selective elimination of SPP1⁺ macrophages could reverse mGGN malignancy. Diverging from the prevailing notion that SPP1⁺ macrophages originate exclusively from monocytes, our data demonstrate that two distinct origins—SPP1⁺ alveolar macrophages (AM) and monocyte-derived macrophages (Mo-Mac)—serve as central players in the malignant evolution of mGGN that progress to early lung adenocarcinoma (LUAD). SPP1⁺ macrophages display functional profiles distinct from FABP4⁺ macrophages and act as regulatory hubs that recruit and reprogram immune cells within the TME. While our results indicate stronger immunosuppressive activity by SPP1⁺ macrophages compared with FABP4⁺ macrophages, the high abundance of FABP4⁺ macrophages in malignant nodules suggests that they also exert regulatory effects on the microenvironment. Notably, the existing literature points to several pro-inflammatory roles for FABP4⁺ macrophages, including enhanced production of pro-inflammatory cytokines that bolster anti-tumor immunity in non-small cell lung cancer (NSCLC) [55] and promotion of M1-like polarization via secreted FABP4 [56, 57]. Furthermore, FABP4⁺ macrophage proportions decline in brain metastases from primary lung tumors and in lung cancer tissues compared with normal lung

[29]. Our flow cytometry data from human pulmonary nodule samples confirmed that FABP4⁺ macrophages express elevated IL-6 and TNF- α , reduced Arg-1 and PD-L1, and display superior phagocytic activity compared to SPP1⁺ macrophages. These characteristics imply that FABP4⁺ macrophages do not promote immunosuppression in the TME and may, in fact, inversely correlate with lung cancer progression.

One proposed model suggests that, upon exposure to stressors such as malignancy, certain monocyte-derived macrophages gradually acquire properties of more adaptable tissue-resident macrophages, especially under prolonged or severe injury [13]. Given the marked reduction (“collapse”) of alveolar macrophages observed during mGGN malignant progression and the established role of alveolar macrophages in pro-tumorigenic niche formation in early lung cancer [11, 58], we propose that, in early-stage disease, monocytes and alveolar macrophages jointly contribute to the anti-inflammatory CD163⁺ SPP1⁺ macrophage pool. These cells localize at the tumor boundary and establish an immunosuppressive barrier through interactions with diverse immune populations. Our findings provide novel evidence that SPP1⁺ macrophages are predominantly monocyte-derived, suggesting that signals from tumor cells or other microenvironmental components drive their recruitment and differentiation. However, the precise mechanisms governing this process remain to be elucidated, representing a limitation of the current work. Our analyses, together with prior reports [59], demonstrate that SPP1⁺ macrophages drive the formation of an immunosuppressive microenvironment and tumor progression in humans. This study is the first to employ SPP1^{-/-} BMDMs in both in vitro and in vivo models to substantiate the critical role of SPP1⁺ macrophages in early LUAD initiation and advancement. Relative to wild-type BMDMs, SPP1^{-/-} BMDMs exhibited impaired responses to IL-4/IL-13-induced immunosuppressive polarization, senescence, and migration, reinforcing earlier links between macrophage senescence and immunosuppressive activity [40, 53]. In vitro co-culture studies showed that SPP1^{-/-} BMDMs failed to enhance CMT64 cell proliferation and migration to the same extent as wild-type BMDMs. Moreover, SPP1-deficient BMDMs were less effective at suppressing CD8⁺ T cell proliferation and IFN- γ release compared to wild-type cells. In vivo xenograft experiments revealed that while wild-type BMDMs robustly promoted CMT64 tumor growth, co-injection of

SPP1^{-/-} BMDMs led to modest inhibition of tumor expansion, with growth rates slightly below those of CMT64-only controls. This reversal likely stems from SPP1 knockout primarily disrupting immunosuppressive polarization while preserving or favoring immune-activating macrophage functions. Consequently, SPP1^{-/-} BMDMs may preferentially adopt anti-tumor phenotypes in the early TME. This interpretation was supported by flow cytometry of tumor-infiltrating immune cells, which showed reduced frequencies of PD-1⁺ CD8⁺ T cells, Tregs, and CD206⁺ macrophages in SPP1-knockout co-injection xenografts compared to controls. In vivo blockade experiments targeting CD8 α and CD44 confirmed that SPP1⁺ macrophages inhibit CD8⁺ T cell function through the SPP1/CD44 axis, thereby accelerating tumor progression. In contrast, SPP1-deficient BMDMs co-cultured with CMT64 cells significantly hampered tumor development in vivo, potentially due to enhanced effector CD8⁺ T cell infiltration in the TME following SPP1 loss. These data suggest that targeted inhibition or genetic deletion of SPP1 in macrophages could reshape the tumor immune landscape and suppress tumor growth.

There are conflicting observations regarding the roles of SPP1⁺ macrophages in cancer. A comprehensive multi-tumor dataset analysis [60] suggested that SPP1⁺ macrophages may possess enhanced phagocytic capacity, engulfing greater numbers of tumor cells. Additionally, higher SPP1⁺ macrophage infiltration has been associated with improved outcomes after immune checkpoint blockade [61, 62]. These discrepancies with our findings may arise from differences in experimental systems and tumor contexts. Further investigation is warranted to clarify the context-dependent mechanisms by which SPP1 regulates macrophage behavior across various tumors and models.

Conclusion

Contrary to the established view that SPP1⁺ macrophages derive solely from monocytes, our study reveals that two distinct populations—SPP1⁺ alveolar macrophages and monocyte-derived macrophages—emerge as key drivers in the malignant progression of mixed GGN that advance to early LUAD. SPP1⁺ macrophages display functional distinctions from FABP4⁺ macrophages and function as central regulators that recruit and reprogram immune cells in the TME. Complementary in vitro and in vivo experiments

established that SPP1 is indispensable for immunosuppressive macrophage polarization. SPP1 knockout in BMDMs reverses the immunosuppressive niche and attenuates LUAD xenograft tumor growth in mice. Collectively, these insights identify SPP1⁺ macrophages as a promising therapeutic target for intercepting the initiation and progression of early-stage LUAD.

Acknowledgments: None

Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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