



Functional remodeling of tissue-resident macrophages leads to distinct immune microenvironment evolution and clinical manifestations in lung adenocarcinoma

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Abstract

Given the indispensable role in extracellular matrix remodeling and immunosuppressive microenvironment formation, the importance of tissue-resident macrophages (TRM) in the occurrence of early-stage cancer has been constantly mentioned. And it is noteworthy that the widespread application of low-dose CT (LDCT) has resulted in a marked increase in the proportion of early-stage patients for lung cancer in recent years, including plenty adenocarcinoma in situ (AIS) patients with ground-glass nodule (GGN) feature on imaging. The group of GGN-like AIS (AIS with ground-glass nodule feature) patients have gradually become a clinical challenge for thoracic surgeons, as surgically removed considered justified only when there is evident malignant progression risk. However, despite many teams, including ours, have proposed possible strategies for non-invasive and efficient assessing the malignant risk of GGN-like AIS patients, the unclear mechanism of the malignant progression for GGN-like AIS toward early-stage lung adenocarcinoma (LUAD) limits further clinical application and optimization. In this study, utilizing transcriptome, we classified TCGA-LUAD patients into distinct TRM functional states and conducted a comprehensive analysis of the physiological significance behind each subtype. Utilizing single-cell data, we have well mapped the results of transcriptome analysis at the cellular level and ultimately identified that *KRT6A*⁺ Ep may be the key epithelial subpopulation for the functional remodeling of TRM. Our study deepened the understanding of the malignant transformation mechanism of GGN-like AIS, as well as provided referential indicators for more personalized treatment and management of LUAD patients.

Keywords Tissue-resident macrophages · Ground glass nodules · Lung adenocarcinoma · *KRT6A* · Immunosuppression

Introduction

Widespread utilization of novel fate-mapping mouse models in cancer research led to the broad recognition of the dual-source characteristics in tumor-associated macrophages (TAM) [1]. Specifically, simply categorizing TAM into M1/M2 states was deemed unsuitable [2–7]. Scholars more tended to classify TAM into tissue-resident macrophages

(TRM) which primarily associated with matrix remodeling and immune regulation, and monocyte derived macrophages (Mo-Mac) which mainly involved in replication response of cancer cell [8]. Compared to Mo-Mac, which only originate from bloodstream and located within the cancer nest [8, 9], TRM exhibits more complexity. For the source, a portion of TRM in tumors derives from resident macrophages with self-renewal and replication abilities that have already colonized each organ during the embryonic yolk sac stage, while another portion arises from monocyte transformation in the early-stage of cancer occurrence with distinct conversion rates observed across different organs [1, 10]. Furthermore, apart from being widely surrounded the cancer foci to remodel the external stromal environment thus promoting cancer invasion and progression [11], TRM also encompasses functional subpopulations situated in specific spatial locations. A previous study on breast cancer revealed *FOLR2*⁺ TRM related to favorable prognosis, situated in

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proximity to tumor blood vessels and associated with $CD8^+$ T infiltration [12]; and the latest researches demonstrated that in lung cancer, a subpopulation of TRM transformed from monocytes specifically colonize near blood vessels and linked to immunosuppression [13–15]. Subsequent research further pointed out that in contrast to the origin, spatial positioning may exert a greater influence on the functional evolution of TRM which requires more in-depth study to confirm [16–20]. Significantly, targeted interventions aimed to specific functional subpopulations of TRM have showed nearly curative effects on cancer in preclinical trials [2–7]. All of these have triggered a surge in exploration on TAM in recent years (sources, functions, spatial positioning, intervention strategies, etc.), as a large amount of TAM-related research from previous decades may have been inadequate and overlooked many critical parts.

In lung adenocarcinoma (LUAD), TRM has been proved to play a crucial and even decisive role in the early-stage of cancer occurrence [8]. And a notable clinical phenomenon is that with the popularity and application of low-dose CT (LDCT), the proportion of early-stage LUAD patients has markedly increased, including large amounts of adenocarcinoma in situ (AIS) patients that predominant exhibit ground glass nodules (GGN) on imaging [21–25]. More importantly, in the latest WHO lung cancer classification system, AIS has been excluded from LUAD and referred to as a precursor glandular lesion [26]. These presents a significant challenge for thoracic surgeons, specifically in assessing and monitoring the malignant risk of GGN-like AIS (AIS with ground-glass nodule feature) transforming into early-stage LUAD during a long-term follow-up, and determining the appropriate timing for surgical intervention. Previously, several research teams, including ours, have proposed some strategies for monitoring the risk of GGN-like AIS malignant transformation [27–29]. However, the specific mechanism underlying the progression of GGN-like AIS to minimally invasive adenocarcinoma (MIA), develops into invasive adenocarcinoma cancer (IAC), and ultimately transformation into early-stage LUAD have not yet been fully elucidated. This impedes the progress of multiple malignant monitoring and intervention strategies to clinical practical application. Therefore, we focus on the functional phenotype remodeling of TRM in the malignant transformation of GGN-like AIS toward early-stage LUAD and preliminarily investigate the impact of the functional remodeling on the immune microenvironment.

In summary, based on the differential expression genes of TRM during the early-stage of LUAD occurrence [8], we classified TCGA-LUAD patients into distinct TRM functional states and conducted a comprehensive analysis of the physiological significance behind each subtype. Utilizing single-cell data, we have well mapped the results of transcriptome analysis at the cellular level and ultimately

identified that $KRT6A^+$ Ep may be the key epithelial subpopulation for the functional remodeling of TRM. Our study deepened the understanding of the malignant transformation mechanism of GGN-like AIS and provided a potential target for intervention strategies in GGN-like AIS malignant transformation, as well as provided referential indicators for more personalized treatment and management of LUAD patients. Compared with previous researches, our work uniquely integrates single-cell data with TRM functional states classification, offering novel insights into the underlying mechanisms and potential therapeutic strategies.

Material and methods

Acquisition and processing of LUAD cohort data

The omics sequencing data and related clinical information of TCGA-LUAD were obtained from the UCSC Xena database. A total of 532 TCGA-LUAD primary patients were enrolled in the analysis. Data for GEE30219 (with 84 LUAD patients), GSE50081 (with 128 LUAD patients) directly acquired from the GEO database. The data processing process were facilitated by Sangerbox tools. And the statistical of clinical feature information could be found in Table 1.

Bioinformatics analysis

Cluster analysis was performed using NMF method, and the average profile width of the common membership matrix

Table 1 Clinical basic information of all databases in this study

Feature	Database		
	TCGA-LUAD	GSE30219	GSE50081
Gender			
Female	197	19	62
Male	200	66	65
Age at diagnosis			
< 60	261	43	19
≥ 60	136	42	108
T stage			
T_{1-2}	346	83	125
T_{3-4}	51	2	2
N stage			
N_0	250	82	94
N_{1-3}	147	3	33
TNM stage			
I	206	None	92
II	98	None	35
III	69	None	None
IV	24	None	None

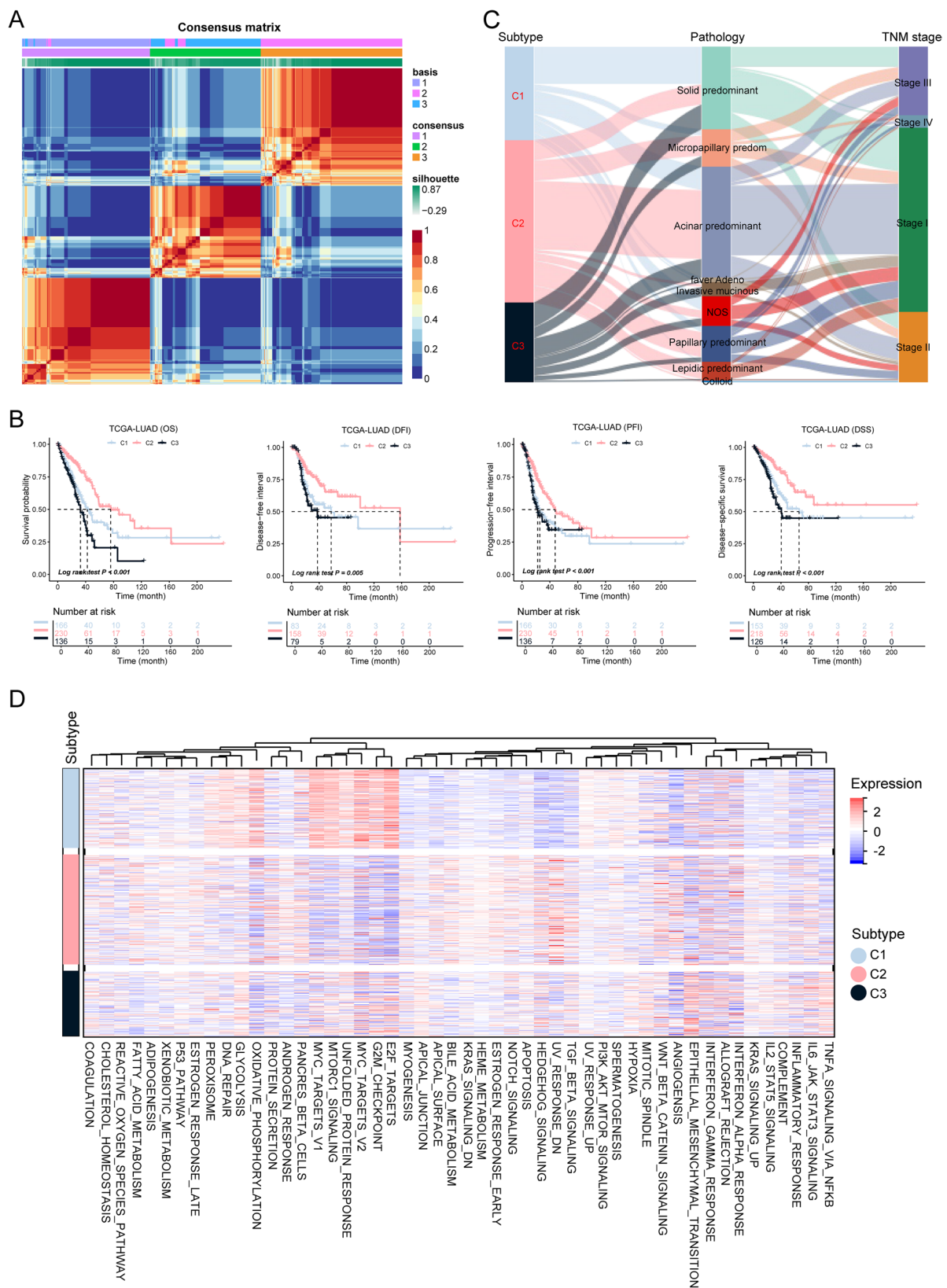


Fig. 1 Identification of potential TRM subtypes of LUAD. **A** Three subtypes were identified as optimal values for consensus clustering by NMF method. **B** Kaplan-Meier curves showing OS, DFI, PFI, DSS of TRM subtypes in TCGA-LUAD. **C** Sankey diagram showed

the correlation between TRM subtypes, stage, and histopathology. **D** GSVA analysis of the 50 hallmark pathways in C1-C3 subtype for TCGA-LUAD patients

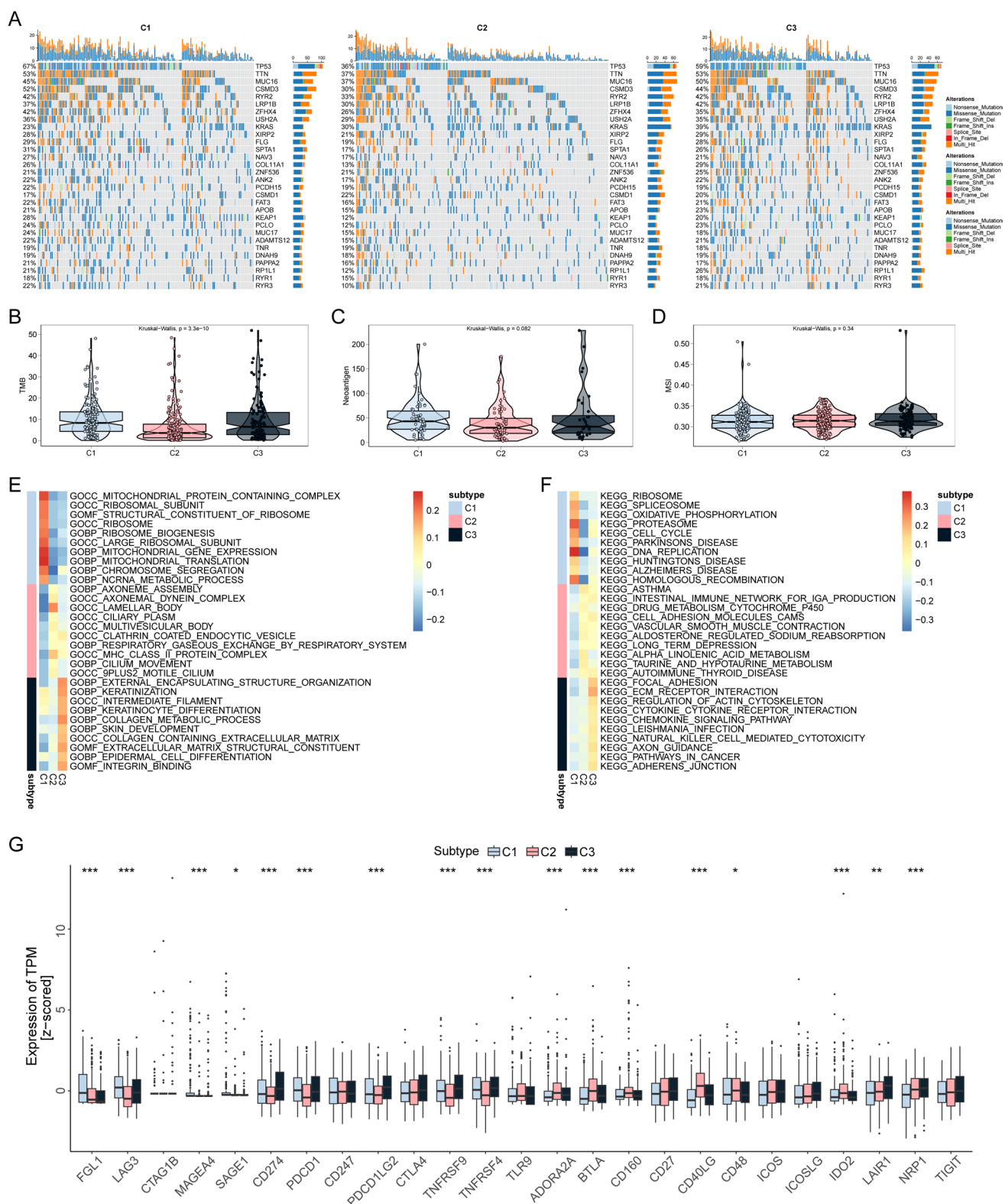


Fig. 2 The evolution of microenvironment in distinct TRM subtypes for LUAD. **A** Top 30 mutation frequency genes in C1-C3 for TCGA-LUAD. Differences in the expression levels of TMB (**B**), NEO (**C**) and MSI (**D**) among the three subtypes. Significant pathways of GO

(**E**) and KEGG (**F**) functional enrichment among the three subtypes. **G** Box plot displayed the differential expression about immune check-point molecule for each TRM subtype

was determined by the R package “NMF”, with the minimum membership of each subclass set to 10. The optimal number of clusters was determined using the cophenetic coefficient plot. GSVA was performed to identify significantly pathway in each subtype, using R package GSVA 1.34.0, on the hallmark gene sets in MsigDB database with default parameters. The limma R package was used for differential expression gene analysis among different subtypes ($\log_{2}FC > 0.585$, adjusted p -value < 0.05), and genes with an expression value of 0 exceeding 50% were removed during the analysis process.

Immune related analysis at transcriptome

Infiltration levels of 28 immune cells in each subtype were assessed using the ImmuneCellAI database [30, 31]. And the TIDE scores of each subtype were obtained through the TIDE database [32, 33]. Relevant process was implemented in R studio (R version = 4.2.3) and visualized with the assistance of GraphPad Prism 9.

Random survival forest analysis

Feature selection was performed using the randomForestSRC package. We also used the random survival forest algorithm to rank the importance of prognostic-related genes ($nrep = 1000$, which indicates that the number of iterations in the Monte Carlo simulation is 1000). We identified genes with relative importance > 0.2 as final marker genes.

Analysis of the scRNA-seq data

We used Seurat package for scRNA-seq data processing [34]. The Stringent filtering for the scRNA-seq was based on the following criteria: (1) cell with a mitochondrial gene proportion exceeding three times the median absolute deviation (MAD) was excluded; (2) cell with detected gene counts < 200 or $> 3 \times MAD$ and UMI counts above three times the MAD was filtered out; (3) cell with ribosomal gene proportion $> 3 \times MAD$ was excluded; (4) doublets were identified and removed using “DoubletFinder” algorithm (version 4), with parameters set as $pN = 0.25$, pK auto-selected based on BCmvn optimal value, and $nExp$ calculated according to sample cell count.

For normalization, the conventional “LogNormalize” method was employed, while the “SCTransform” method was applied for the identification of macrophages subpopulations to stabilize variance and correct technical biases.

For batch effects in large-scale scRNA-seq studies, the “Harmony” algorithm was utilized to integrate data across different cohorts or sequencing batches by correcting batch-specific variations.

Identification of macrophage subpopulations

To identify macrophage subpopulations at the single-cell level, the “Louvain clustering” algorithm was employed. The optimal number of clusters was determined via evaluating the modularity score and stability of clustering results across a range of resolutions. Ultimately, nine distinct macrophage-related clusters were identified, each characterized by unique gene expression profiles. Based on prior reported [8], four TRM marker genes (MARCO, MRC1, STMN1, PPARG) were examined in the nine clusters to further identify TRM subpopulation, with Cluster 4 and 5 designated as TRM and the remaining classified as Mo-Mac.

For dimensionality reduction, principal component analysis (PCA) was applied to capture dominant sources of variation in the dataset. The top 50 principal components ($npcs = 50$) were selected based on cumulative variance, ensuring retention of substantial data variability. Subsequently, Uniform Manifold Approximation and Projection (UMAP) was used for visualization, leveraging the first 20 principal components ($dims = 1:20$) to balance global structure preservation and local cluster resolution.

Pseudotime trajectory analysis

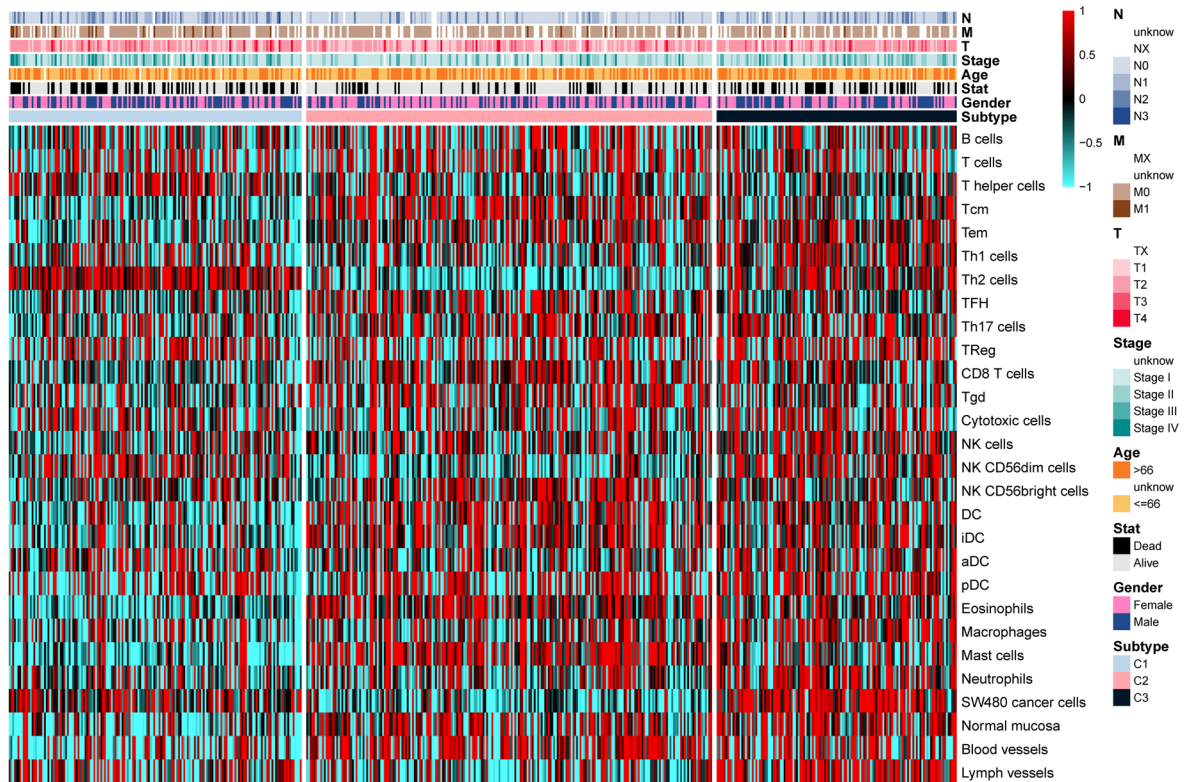
Single-cell pseudotime trajectories were constructed using the Monocle 2 package [35]. The trajectory analysis was performed on highly variable genes identified using the function “dispersionTable”. Then the “reduceDimension” (method = “DDRTree”) function was applied to reduce dimensions and the “orderCells” function was used for construction of pseudotime trajectory and alignment of cells within the pseudotime time. Besides, the function “plot_cell_trajectory” was employed to display the pseudotime trajectory.

Analysis of the spatial RNA-seq data

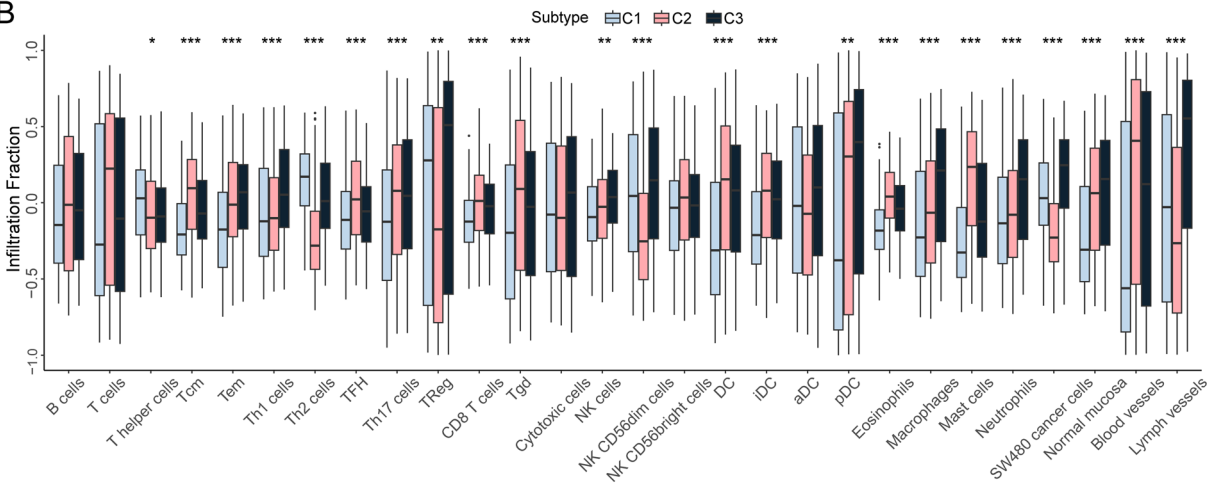
In GSE189487, spatial transcriptomic profiling was performed at the tissue section level using the $10 \times$ Genomics Visium platform, with per-spot spatial resolution. Each spot comprises multiple cells, thus reflecting regional rather than single-cell resolution. To evaluate the spatial proximity of tissue-resident macrophages (TRM) to KRT6A + epithelial cells, the FeaturePlot function with “spatial” reduction was employed to visualize spatial expression of FGL1 (CM1), BIRC5 (CM2), SFTPC (CM3), and KRT6A. By integrating spatial expression signals with histological images, colocalization patterns of these cell populations in tissue were inferred.

For spatial clustering and interpretation, unsupervised spatial clustering was conducted via sequential application of SCTransform normalization, principal component

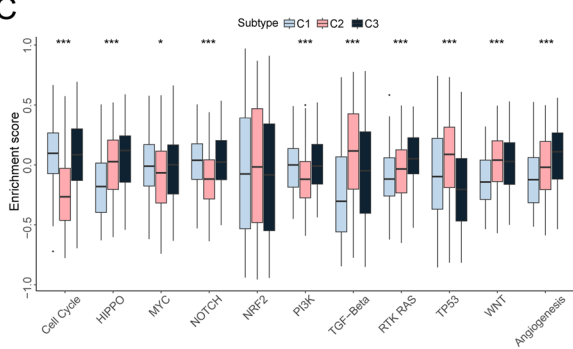
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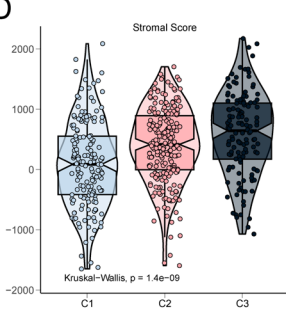
B



C



D



E

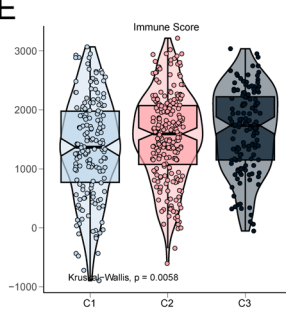


Fig. 3 Analysis of the differential immune cell infiltration among the three subtypes. **A** Heatmap showed the differential infiltration of 28 immune cell types in TCGA-LUAD patients under ssGSEA algorithm. **B** Box plot displayed the differential infiltration about 28 immune cell types for each TRM subtype. **C** Differential activation levels of 11 carcinogenic pathways among three subtypes. The analysis of stromal score (**D**) and immune score (**E**) among three subtypes under estimated algorithm

analysis (RunPCA), nearest neighbor identification (FindNeighbors), and clustering (FindClusters, resolution = 0.2). Clustering results were visualized via DimPlot and SpatialPlot to depict spatially distinct regions. TRM-enriched areas were identified by integrating cell subpopulation annotations (e.g., first_type, second_type) and signature gene expression (e.g., FGL1, BIRC5, SFTPC), enabling delineation of cellular composition and functional states across regions.

Furthermore, robust cell type decomposition (RCTD) was utilized for spatial transcriptomic deconvolution [36]. This involved construction of a high-quality scRNA-seq reference (GSE189357) and generation of a reference object using its annotation information. Spot-level expression matrices were fed into the RCTD model to derive cellular composition proportions, with primary cell type annotations assigned to each spot based on second_type and first_type fields. TRM-enriched regions were specifically defined as spots with dominant TRM proportions in RCTD deconvolution results.

Cell–cell communication analysis

We employed the CellChat package to infer communication networks [37]. We used the default CellChatDB as the ligand–receptor database and identified significant ligand–receptor interactions based on overexpressed ligand or receptor as well as P -value < 0.01. The “netAnalysis_signalingRole_heatmap” and “netVisual_bubble” were utilized to display signaling differences and specific ligand-receptor differences among the cell types.

Statistical analysis

SPSS 26 and Graphpad Prism 9 were used for the statistical analysis. When the two groups of measurement data are compared and the variance is homogeneous, the Student's t -test was used. If the variance is not homogeneous, the Mann–Whitney U test was used. Chi-square test or Pearson precision test was used for comparing the counting data. Survival analysis was performed using the Kaplan–Meier method. P values less than 0.05 ($P < 0.05$) was considered statistical significance.

Western blot

Cells under different conditions were lysed on ice with RIPA lysis buffer (Sangon Biotech), followed by quantification of their protein concentrations through the bicinonic acid (BCA) protein assay kit (Beyotime Biotech). Protein sample was separated by electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel and subsequently transferred to an Immobilon PVDF membrane. The membrane was incubated with primary antibody (Rabbit anti-KRT6A, ab93279, abcam) for a night at 4 °C and then gently shaken with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG-HRP, sc-2004, Santa Cruz Biotechnology). The bands were soaked with chemiluminescence detection buffer (Takara, Shiga, Japan) and its intensity was detected by the corresponding software.

Results

Differential functional remodeling of TRM corresponds to distinct outcomes

Functional remodeling of TRM has been widely proven to play a crucial role in cancer occurrence and development [38, 39]. Hence, we sought to investigate whether the typing on TRM functional states could be employed to identify high-risk individuals among LUAD patients. In a previous study utilizing spontaneous mouse model of LUAD [8], we obtained 1322 differentially expressed genes in TRM of early-stage tumorigenesis (15 days) compared to that in normal lung tissue, and 1157 genes ultimately used for TRM functional typing in Homo sapiens owing to the differences between species. Considering the cophenetic coefficient in NMF typing (Fig. S1A), we clustered 532 TCGA-LUAD patients into three TRM functional states and designated as C1–C3 (Fig. 1A). As expected, there were indeed notable prognosis differences among the three subtypes, C2 associated with a favorable survival while C1 and C3 clearly linked to an adverse outcome (Fig. 1B). Similar results were validated in two publicly available LUAD databases, GSE30219 and GSE50081 (Fig. S1B–E). Interestingly, not limited by the histopathology, the Sankey diagram revealed that TRM in C2 state consistently restricted the LUAD progression to I stages while C1 state continues to drive LUAD progression until III–IV stages (Fig. 1C).

GSVA analysis further demonstrated an opposition distribution of the 50 Hallmark pathway scores in C1 and C2 (Fig. 1D). C1 has the highest score in several carcinogenic pathways (E2F targets, G2M checkpoint, MYC targets V2,

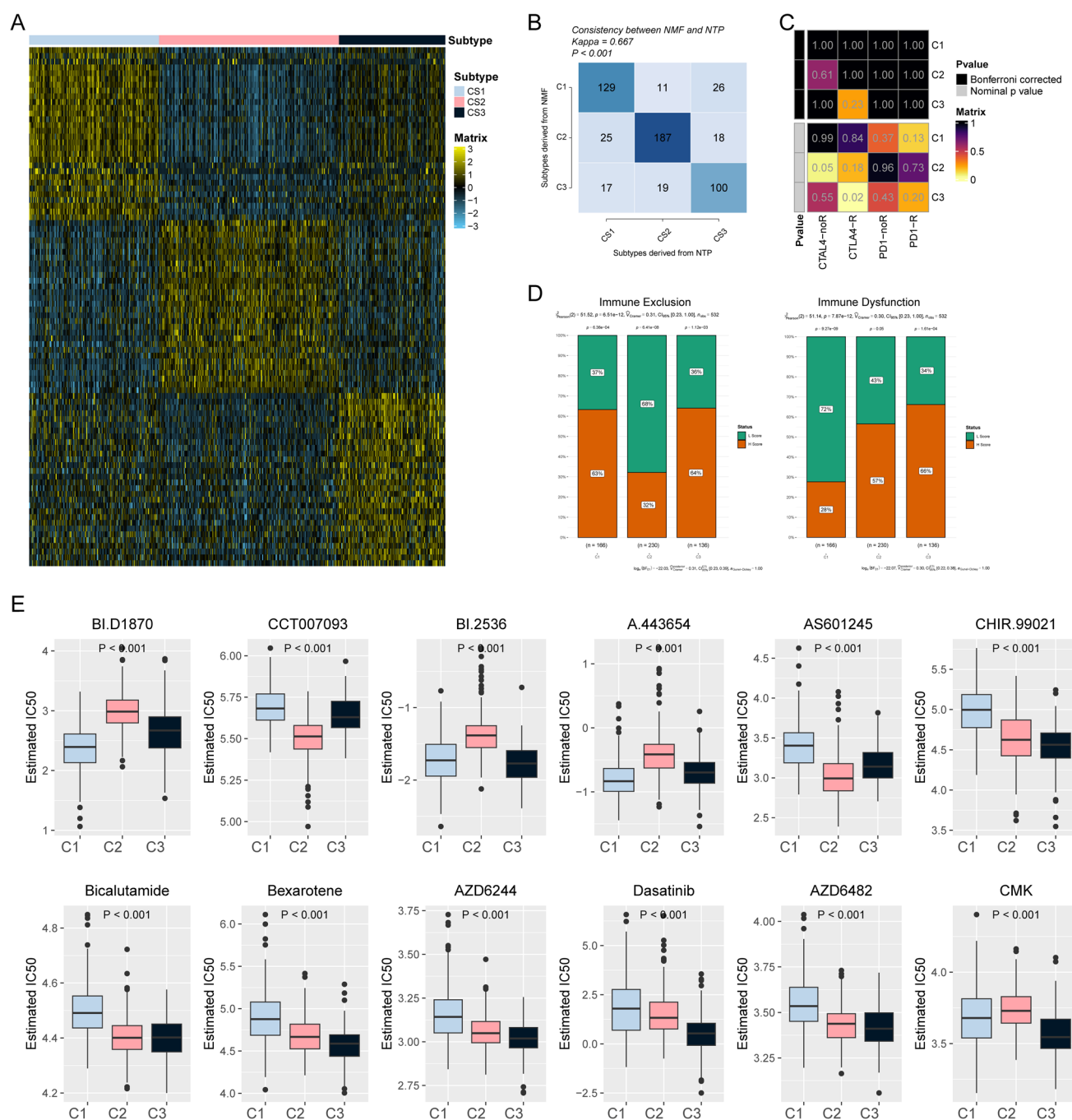


Fig. 4 Immunotherapy prediction and drug sensitivity analysis for TRM subtypes. **A** The heatmap showed the top 30 genes with the largest log2FC value among the differentially expressed genes of each subtype. **B** NTP algorithm was used to predict the subtype of genes in the immunotherapy data set (CS1-CS3) based on 90 features genes

and compare the similarity with the original subtype (C1-C3). **C** Heatmap showed the possible response prediction of the three TRM subtypes to immunotherapy. **D** TIDE score analysis for the three subtypes: Immune exclusion (left), Immune dysfunction (right). **E** Drug sensitivity prediction for the three TRM subtypes

MTORC1 signaling, MYC targets V1 and oxidative phosphorylation), and oxidative phosphorylation has been demonstrated to have a strongly correlated for the maintenance of anti-inflammatory phenotype in macrophages [40].

Distinct subtypes represent diverse evolutions of immune microenvironment

Given the extensive regulatory capacity of TRM on tumor immune microenvironment [10, 41], we focus on exploring

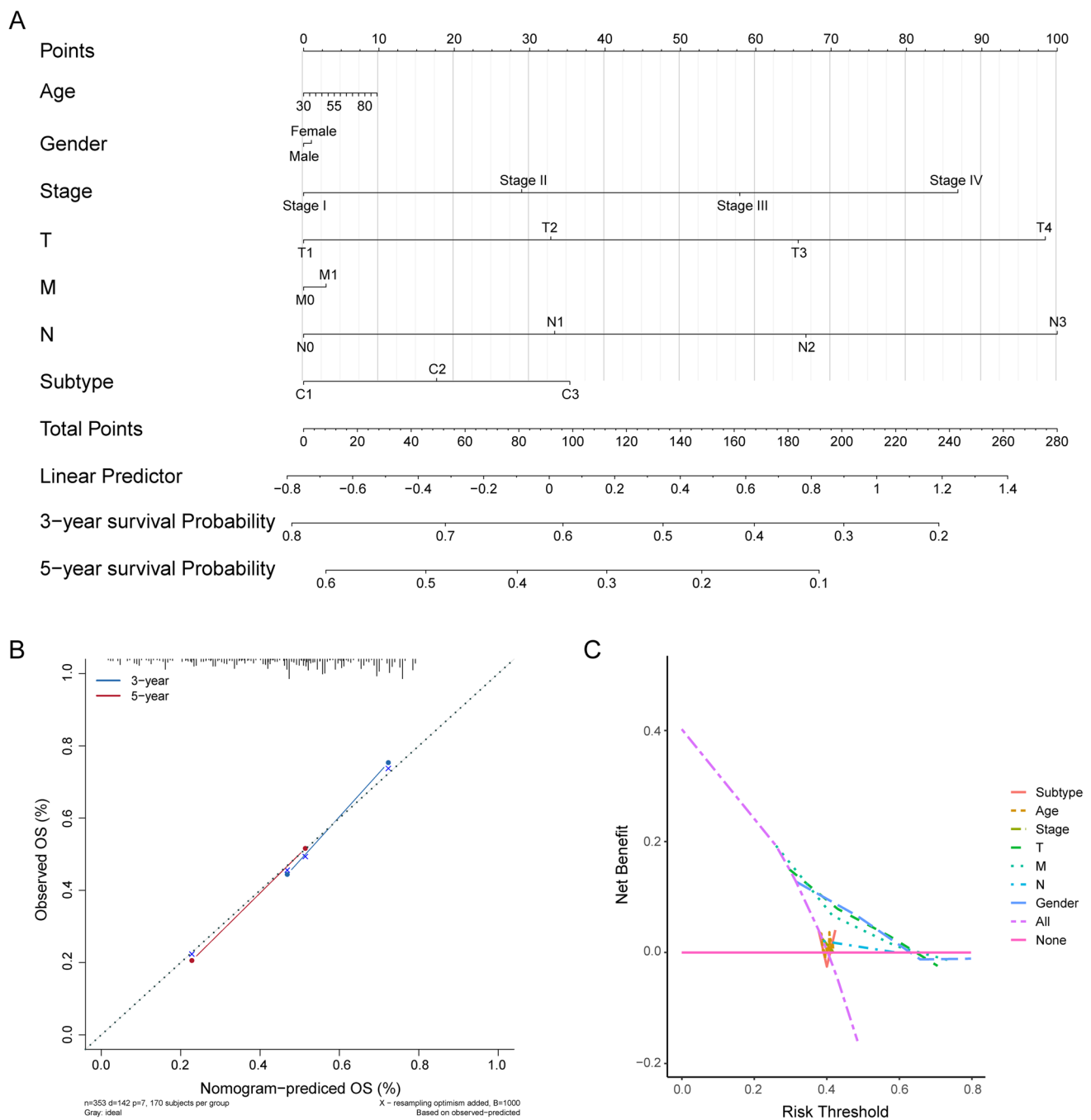


Fig. 5 Prognostic predict value of TRM typing in patients with LUAD. **A** Nomogram construction based on age, gender, stage, T, N, M and TRM subtype in TCGA-LUAD. **B** Comparison of consistency

between nomogram-predicted 3-year and 5-year survival rates and true survival rates. **C** Net benefit plot displayed the predict value for each indicator

the immune environment differences behind the three functional states of TRM. We first explored the top 30 genes with mutation rates in each subtype (Fig. 2A). It could be intuitively observed that top 30 mutated genes of the three subtypes overlapped, but the average mutation rate of C2 was the lowest (average 20.97%), C1 was the highest (average 29.37%), and C3 located in the middle. Consistent with this,

patients classified as C2 subtype exhibited the lowest levels of TMB, NEO, and MSI, while C1 subtype displayed the highest, followed by patients in the C3 subtype (Fig. 2B–D). GO and KEGG functional enrichment further supported the anti-inflammatory phenotype of C1 subtype (high scores of mitochondrial-associated oxidative phosphorylation energy metabolism) and the typical anti-tumor immune response

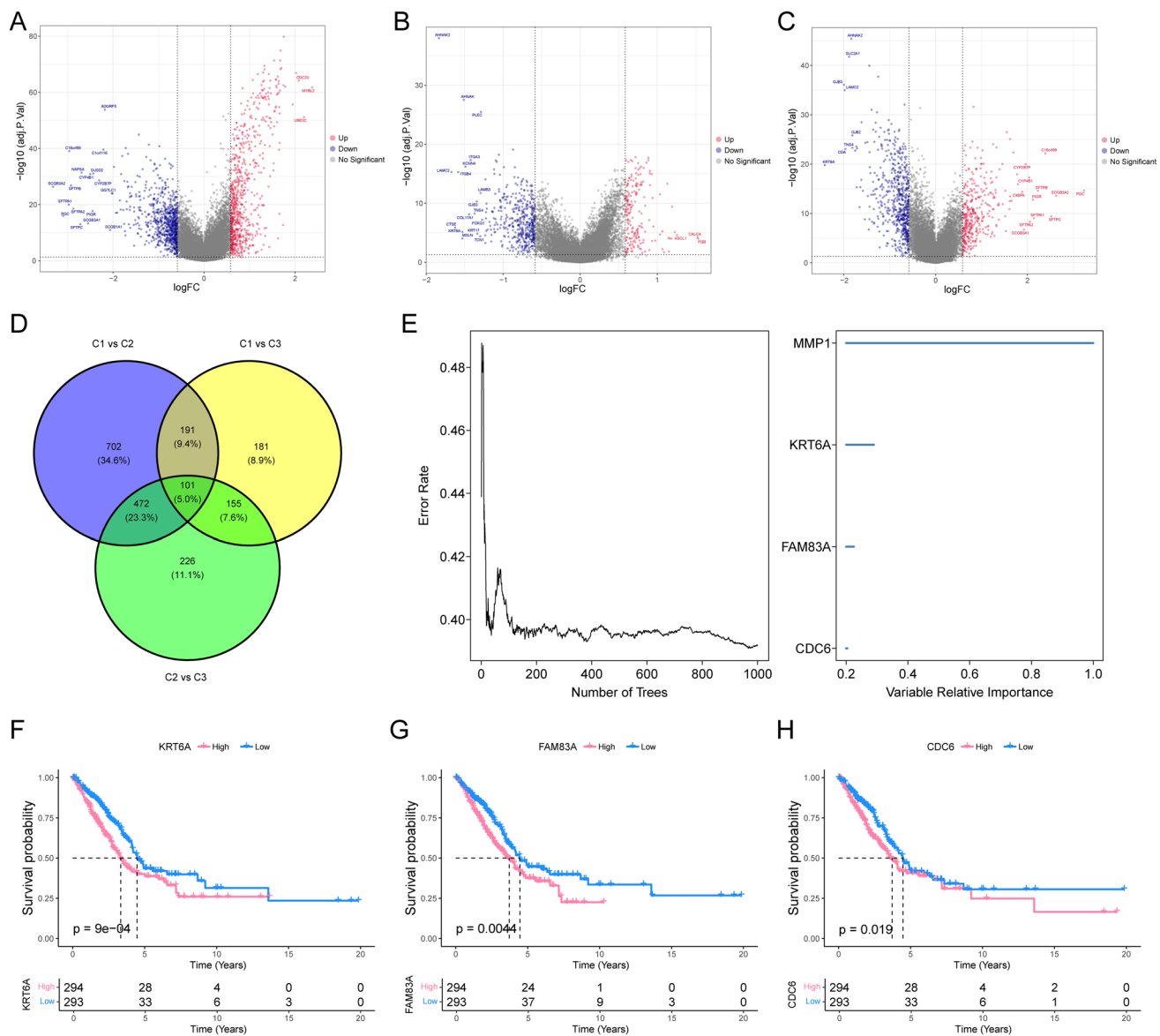


Fig. 6 Identification of key genes for TRM typing. Volcanic map showed the differential genes among the three subtypes: C1 vs C2 (A), C1 vs C3 (B), C2 vs C3 (C). D Using Venn diagram to obtain the intersection of different expression genes among the three differ-

ent subtypes. E Screening the 101 genes by random forest algorithm. The three key genes: KRT6A (F), FAM83A (G), CDC6 (H) identified with prognostic differences

represented by C2 subtype (high score of anti-tumor immune pathway) (Fig. 2E–F). For these, we examined the expression differences of 25 immune checkpoint molecules across the three subtypes (Fig. 2G). Meaningful, while most immune checkpoint molecules have the lowest expression in C2 subtype, the highest expression was not found in C1 subtype, but rather in C3 subtype.

To elucidate the underlying causes of this phenomenon, we employed ssGSEA algorithm to further employ the differences infiltration of 28 immune cell types among the three subtypes (Fig. 3A). The results indicated that C2 subtype was characterized by a high infiltration of multiple

anti-tumor immune cell (Tcm, Tem, TFH, Tgd, etc.), while C3 subtype had a high infiltration of several immunosuppressive cell (TReg, pDC, Th17). In contrast, nearly all immune cell showed the lowest infiltration level in C1 subtype (Fig. 3B). These suggested that C1 subtype represented a “desert” subtype without immune cell infiltration, whereas C3 was categorized as an “immunosuppressive” subtype marked by significant immunosuppressive cell infiltration. This could also explain why in Fig. 1C, C2 subtype were predominantly composed of patients in II stage, yet they exhibit a similar poor prognosis as patients in C1 subtype. And more, the three TRM subtypes also showed significant

differences in the 11 carcinogenic pathways (Cell Cycle, Notch, PI3K, TGF-Beta, TP53, WNT), indicating the possible direction for further exploration of its internal mechanism (Fig. 3C). Consistent with the above findings, C1 subtype has the lowest stromal and immune scores, which further proved its “desert” phenotype of immune infiltration (Fig. 3D–E).

The role of TRM typing in personalized therapy and prognostic prediction

We used NTP algorithm to compare the expression profile of the three TRM subtypes we identified in comparison to another published dataset containing 47 patients with melanoma that responded to immunotherapies [42] (Fig. 4A–B). Results showed that LUAD patients with the C3 subtype may be suitable for immunotherapy, whereas individuals in C1 subtype exhibited a lack of response to immunotherapy (Fig. 4C). TIDE analysis also indicated that C2 subtype had the lowest immune cell infiltration exclusion, while the C1 and C3 subtypes demonstrated comparable rates of immune cell infiltration exclusion; however, C3 subtype had the highest score related to immune cell dysfunction (Fig. 4D). These further substantiated that TRM of C1 subtype blocks the infiltration of almost all immune cells, while TRM of C3 subtype leads to a high infiltration level of suppressive immune cell. In order to identify potential therapeutic agents that may be suitable for LUAD patients with C1 subtype, we conducted a drug sensitivity prediction and found that BI.D1870 and A.443654 as promising candidate candidates (Fig. 4E).

Furthermore, we examined the prognostic value of TRM typing as an independent predictor in LUAD. Nomogram revealed that TRM typing carried a substantial prognostic predictive weight (Fig. 5A). And calibration curves indicated that the nomogram-predicted probability highest matched the actual 3-year and 5-year survival (Fig. 5B). In general, TRM typing could be used as an auxiliary index to better predict the prognosis of LUAD patients (Fig. 5C).

Identification of key genes for TRM typing

To underlying the possible mechanism behind the functional remodeling of TRM into these three subtypes, we first identified their key genes. We analyzed the differentially expressed genes among the three TRM subtypes by volcano map ($\text{adj.}P\text{-Val} < 0.05$ and $|\log\text{FC}| > 0.585$) (Fig. 6A–C), and obtained 101 final candidate genes by utilizing a Venn diagram to determine the intersection (Fig. 6D). Then, we performed a random survival forest analysis on the 101 differential genes and determined the genes with relative importance as the final markers, illustrating the importance ranking of the four genes (Fig. 6E). A survival analysis was

conducted on the four highly critical genes, and the results showed that three of them exhibited significant (Fig. 6F–G). For these, *CDC6*, *FAM83A*, and *KRT6A* will serve as key genes for our subsequent study.

Validation of TRM typing in single-cell data

Only depletion of TRM in the early-stage could inhibit the progression of LUAD [8], and the function of TRM was more likely to undergo profound remodeling during the early-stage then tended to stabilize in the later [2]. A previous study performed a comprehensive analysis on the spatiotemporal transcriptome of GGN-like AIS malignant transformation toward early-stage LUAD (MIA and IAC) [43], which offers valuable resources for us to deeper investigate the relationship between our TRM typing and the early-stage TRM functional remodeling.

We first annotated all cells (after quality control, 88,002 cells were enrolled) as ten distinct populations (Fig. 7A–B), and investigated the proportion changes of each population with the malignant progression (Fig. 7C). Results showed that the proportion of macrophages exhibited a pattern of initial decrease followed by an increase, which was consistent with the previous conclusion [8]. And the metabolic pathway scoring results based on the TCGA-LUAD expression profile revealed that the three TRM subtypes had the most significant differences in lipid metabolic pathways (Fig. 7D). This further supported us to verify the aforementioned transcriptome results at the single-cell level.

Based on these, we performed a detailed subclassification for the macrophage population, and annotated the cluster with high expression of TRM marker as TRM subpopulation, while the remaining clusters as Mo-Mac subpopulation (Fig. 8A–B). With the progression of tissue malignancy, the proportion of Mo-Mac cells gradually increased, whereas TRM showed a gradient decline (Fig. 8C). We further conducted a pseudotime trajectory analysis on TRM, and yielding encouraging results (Fig. 8D–F). TRM was further classified into three states (CM1–CM3), with a marked decline in the proportion of CM3 observed during malignant progression, while the proportions of CM1 and CM2 demonstrated an increase in the final progression to IAC compared to AIS (Fig. 8F). This leads us to speculate that CM3 may represent a cellular mapping of the C2 subtype identified in the transcriptomic analysis, while CM1 and CM2 correspond to C1 and C3. GSVA analysis and characteristic gene expression analysis further supported our conclusion (Fig. 8G–H). The top ten characteristic genes of the C2 subtype in transcriptome were significantly overexpressed in CM3, which also obtained the lowest scores across nearly all 50 hallmark pathways. Besides, top ten characteristic genes of the C1 subtype were observed to be highly expressed in CM2, and CM2 obtained the highest scores in the normal



Fig. 7 Clustering for single-cell data in early-stage LUAD. **A** UMAP plots showed distributions of 10 cell types (left) and three pathological stages (right). **B** Marker gene expression profile for 10 cell types. **C** The fraction of 10 cell types in three states. **D** Heatmap showed the difference of metabolic pathway activity among the three subtypes

immune function pathway (interferon alpha response, interferon gamma response), whereas CM1 obtained the lowest scores which suggested the dysfunction in its immune microenvironment. Overall, the C1 subtype was identified as CM2 at the cellular level, the C2 subtype as CM3, and the C3 subtype as CM1.

Moreover, three previously identified key genes were found to be specifically expressed in epithelial cells (Fig. 8I). Thus, epithelial cells were further subdivided into nine clusters, we found that three key genes were significantly expressed in cluster 2, with *KRT6A* gene being exclusively expressed in cluster 2 (Fig. 8J). Consistent with the biological significance, the proportion of *KRT6A* + Ep gradually increased with the malignant progression (Fig. 8K).

Validation the regulation of *KRT6A* + Ep on the functional evolution of TRM

To deeper insights into the molecular mechanism behind the remodeling of TRM to three functional states, we conducted a cell communication analysis. Results showed that the *RETN-CAP1* signaling pathway, which communicates with T cells, may be the key in the immune dysfunction microenvironment caused by CM1 (Fig. 9A). Meanwhile, the aberrant expression of *LGALS9*, an immune checkpoint related ligand, may play an important role in the differentiation of TRM into three distinct functional states.

More importantly, we pay more attention to the mechanism by which the key epithelial subpopulation *KRT6A* + Ep leads to the evolution of different functions for TRM. Results of cell communication indicated that the enhancement of *MDK*-related pathway and the decrease of *SCGB3A2-MARCO* pathway may be the main factors (Fig. 9B). Furthermore, the results of spatial transcriptome confirmed the regulatory effect of *KRT6A* + Ep on TRM. With the malignant progression, the TRM subtype represented by CM2, which restricted the infiltration of immune cells, gradually transitions from being uniformly dispersed in lung tissue to tightly surrounding the cancer nest (*KRT6A* + Ep), whereas the TRM subtype of normal anti-tumor immune response represented by CM3 evolves from surrounding the cancer nest (*KRT6A* + Ep) to becoming isolated by the cell population represented by CM2 (Fig. 9C).

The phenomenon of *KRT6A*-induced malignant remodeling of TRM was preliminarily verified in vitro. *KRT6A* gene was knocked down in A549 cells using si-RNA, and the knockdown was confirmed by western blot (Fig. 9D).

Subsequently, WT and KO-A549 cells were co-cultured with macrophages derived from THP-1 cells for 24 h, and the expression of classical pro-inflammatory and anti-inflammatory markers on the co-cultured macrophages was detected by RT-qPCR. Results showed that *KRT6A* knockdown indeed significantly increased the activation of pro-inflammatory markers (IL-6, IL-1 β , TNF- α) on macrophages in the co-culture (Fig. 9E), while the activation of anti-inflammatory markers (TNF- β , Arg-1, IL-10) was significantly inhibited (Fig. 9F).

Discussion

The malignancy of cells arises from their own aberrant mutations, yet their progression into cancer requires a substantial accumulation of abnormal mutations [44–46]. Recent study has pointed out the pivotal role of TRM in establishing peripheral immunosuppressive environments during early-stage cancer occurrence, thus expediting the accrual of abnormal mutations [47]. Based on prior research and utilizing data from TCGA-LUAD [8], we clustered the TRM throughout the development of LUAD into three distinct subtypes, and revealed the notable disparities across them in patient clinical outcomes, biological phenotypes and immune environment. Overall, TRM may undergo functional remodeling in three different directions during the development of LUAD: C1 subtype representing the “desert” phenotype that limits the infiltration of all immune cells, C2 subtype epitomizing the normal anti-tumor immune response, and C3 subtype promotes the infiltration of a substantial number of immunosuppressive cells which characterized as the immunosuppressive environment.

We further explored the guiding role of TRMs typing in personalized diagnosis and treatment management for LUAD patients. Consistent with immune cell infiltration results, C1 subtype is not suitable for immunotherapy, with A.443654 and B1.D1870 as potential candidate drugs. The C2 and C3 subtypes showed responsiveness to CTLA-4 therapy, corroborating the strongest infiltration correlation between TRM and Treg cells during cancer development [8]. However, the C3 subtype may be more suitable for treatment with CTLA-4 inhibitors due to its significantly high Treg cells infiltration. The C2 subtype, in contrast, may not benefit from CTLA-4 inhibitors (treatment response significance: CTLA4-noR vs CTLA-R = 0.05 vs 0.18). Given that excessive reduction of Treg cell infiltration around cancer increases the risk of immune-related adverse events (irAEs), including polymyositis (PM) and dermatomyositis (DM) [48, 49]. The C2 subtype is more likely to select patients who are relatively unsuitable for CTLA-4 inhibitor treatment due to moderate Treg cell infiltration in their

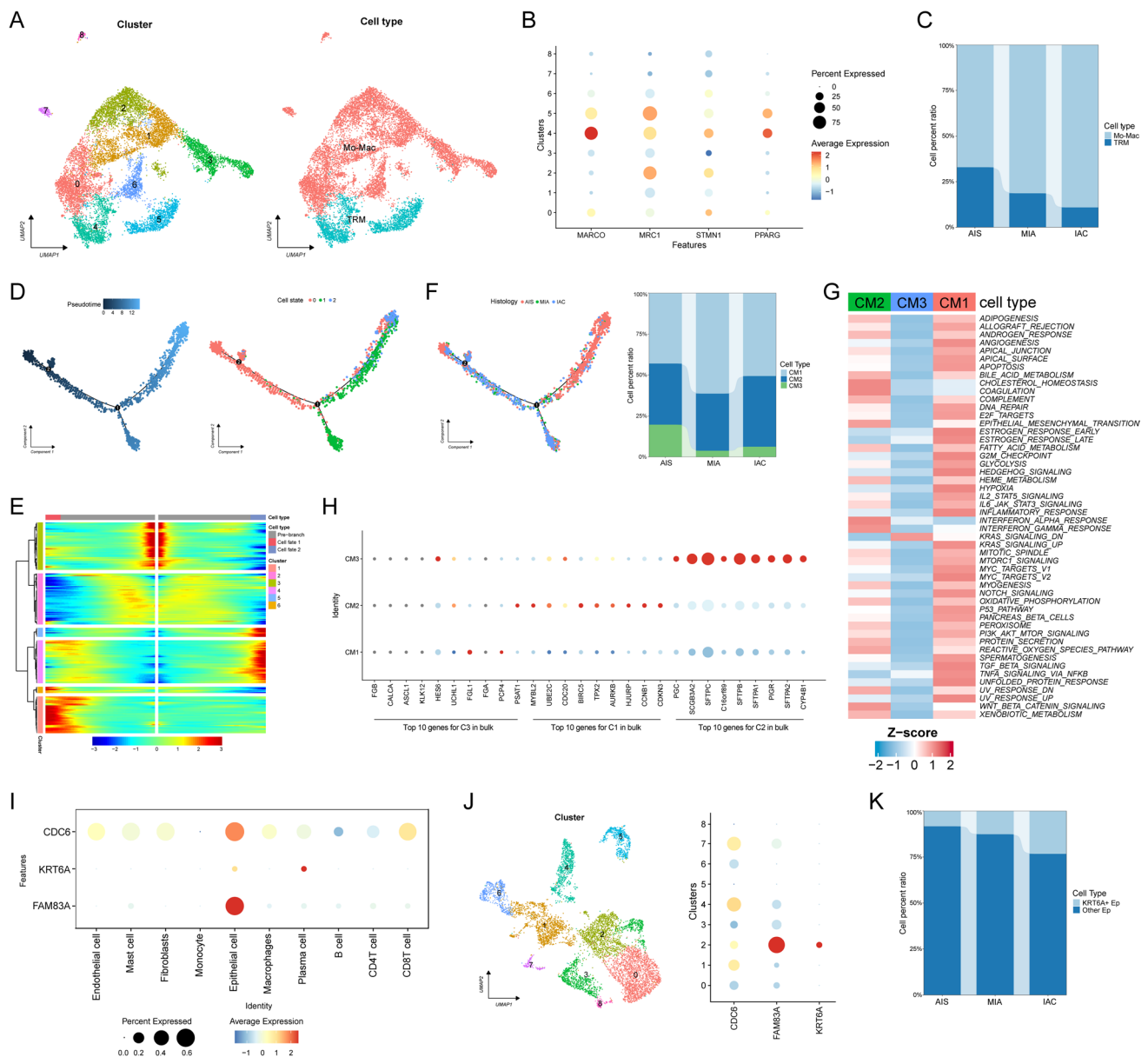


Fig. 8 Verification the TRM typing from transcriptome in single-cell data. **A** UMAP plots showed distributions of macrophages reclustering: cluster (left) and cell type (right). **B** Marker gene expression profile of TRM for nine macrophage clusters. **C** The cell ratio changes of TRM and Mo-Mac in three pathological stages. **D** Pseudotime trajectory analysis for TRM in single-cell data. Left: The pseudotemporal progress of TRM cell differentiation; Right: TRM cells color-coded by different states (state 0, 1, 2). **E** Heat map depicting the genes with significant differential expression of TRM cells in predicted direction of cell differentiation. **F** Left: Distribution of cells from three patho-

logical stage in three pseudotime states. Right: The dynamic changes of the three states of TRM (CM1, CM2, CM3) with the malignant progression of early-stage lung cancer. **G** Heatmap showed the difference of 50 hall mark pathway activity among three TRM states. **H** Expression of top 10 characteristic genes about TRM subtypes (C1-C3) in three TRM cell states (CM1-CM3). **I** Expression of key genes in 10 cell types. **J** Expression of three key genes in nine epithelial clusters. **K** Changes in the proportion of *KRT6A*+Ep with malignant progression

microenvironment. And this still requires careful validation in subsequent clinical cohorts.

To investigate the specific functional subpopulations of TRM associated with the three subtypes at the cellular level, we used previously published spatiotemporal transcriptomic

data on the evolution of GGN-like AIS progressing toward MIA and ultimately to IAC for in-depth exploration [43], and the results were highly encouraging. After a pseudotime trajectory analysis of TRM, we identified distinct differentiation states (CM1-CM3) of TRM at the cellular level, and

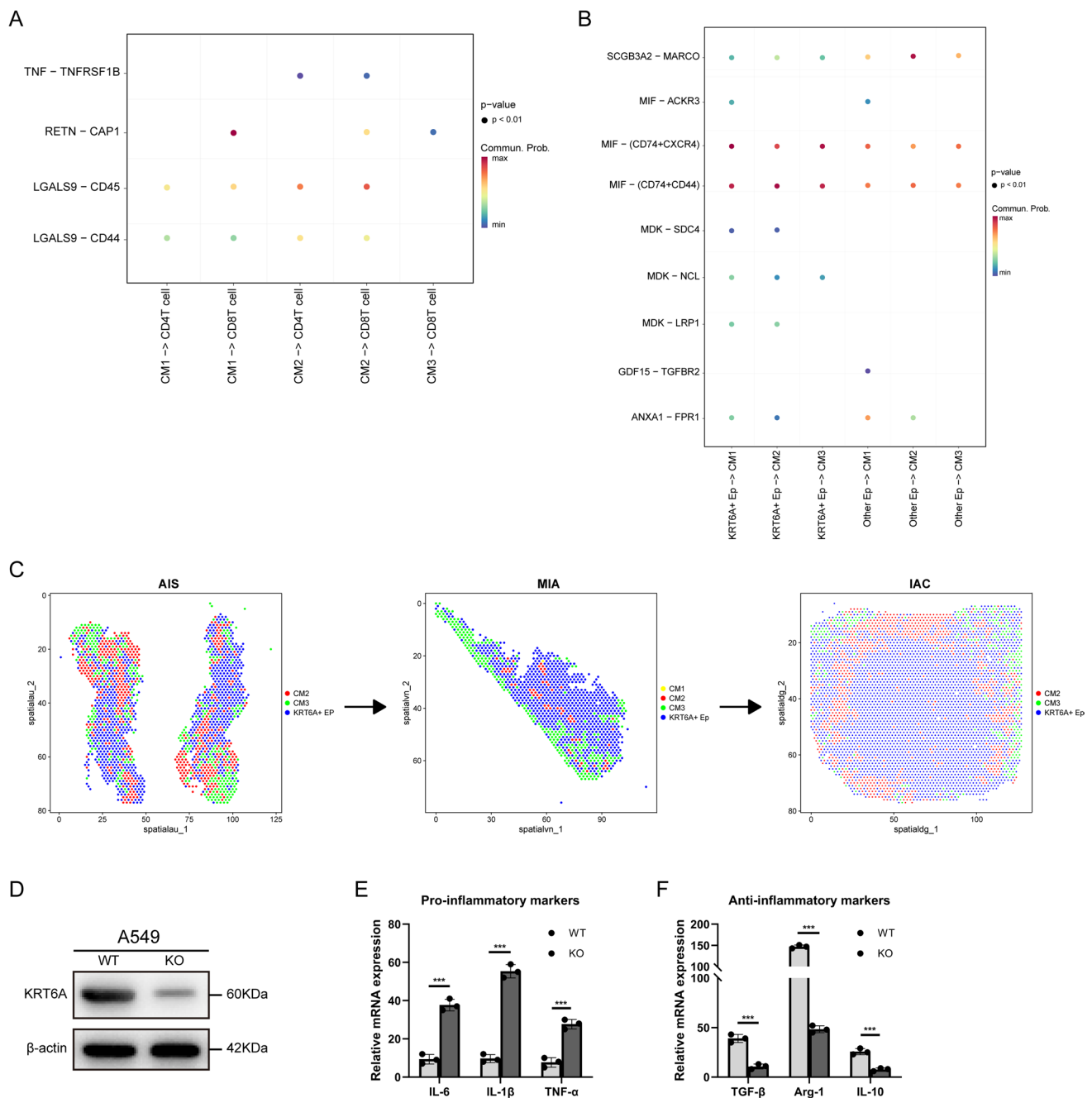


Fig. 9 Regulation of TRM functional remodeling by *KRT6A* + Ep and validation in spatial transcriptomics. **A** Analysis of the cell communication between three TRM functional states and CD8 + T, CD4 + T cells. **B** Analysis of the communication between three TRM functional states and *KRT6A* + Ep, other Ep. **C** Identification the spatial position change about *KRT6A* + Ep and three TRM functional states

(CM1–CM3) with the malignant progression in spatial transcriptome. **D** Knockdown of the *KRT6A* gene in A549 cells via si-RNA verified by western blot. Expression of pro-inflammatory (**E**) and anti-inflammatory (**F**) markers on the macrophages co-cultured with WT or KO-A549 cells for 24 h detected by RT-qPCR

well matched the three subtypes (C1–C3) of TRM in transcriptome through joint function analysis and characteristic gene analysis. Our results further elucidated that the key mechanism underlying the immunosuppressive phenotype of CM1 state (corresponding to C3 in the transcriptome)

may be the abnormal enhancement of *RETN-CAP1*, which has recently been confirmed to be strongly related to inflammatory response [50, 51].

And more, we also conducted a preliminary exploration about the mechanism underlying the function evolution of

TRM to three distinct functional states. At the transcriptome level, we initially examined the differentially expressed genes among the three TRM subtypes (C1-C3) and identified three candidate genes (*CDC6*, *FAM83A*, and *KRT6A*) by random forest algorithm. Interestingly, these three genes were specifically expressed in lung epithelial cells at the cellular level, and *KRT6A* being expressed exclusively in a specific subpopulation. This is also consistent with the current understanding that epithelial cell lines are thought to have extensive and abundant cross-linking with TRM in the early stage, thus facilitating cancer progression [52]. Meanwhile, the up regulation of *KRT6A* gene was considered to be strongly associated with a highly plasticity state in lung cancer cells in recent studies [53, 54]. For these, we designated *KRT6A* + Ep as the critical epithelial subpopulation involved in regulating the functional remodeling of TRM. The results of cell communication suggested that the down-regulation of *SCGB3A2-MARCO* pathway may be the key mechanism underlying TRM function remodeling, as *SCGB3A2* is considered to be related to non-cancer origins epithelium (type I alveolar epithelium) [55]. Spatial transcriptome further confirmed the possible regulation of *KRT6A* + Ep on TRM functional remodeling. With the malignant progression, the TRM subtype represented by CM2, which restricted the infiltration of immune cells, gradually transitions from being uniformly dispersed in lung tissue to tightly surrounding the cancer nest (*KRT6A* + Ep), whereas the TRM subtype of normal anti-tumor immune response represented by CM3 evolves from surrounding the cancer nest (*KRT6A* + Ep) to becoming isolated by the cell population represented by CM2.

Conclusion

Overall, our results showed that TRM has three distinct functional states in the development of LUAD, each significantly representing different biological states, tumor micro-environment and clinical outcomes. And the cross-linking of *KRT6A* + Ep with TRM through *SCGB3A2-MARCO* may play an important role in the early functional differentiation of TRM. Our findings deepened the understanding of the malignant transformation mechanism of GGN-like AIS and provided referential indicators for more personalized treatment and management of LUAD patients.

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Author contributions YL and JD conceived and designed the study. YL and LJ performed the research and analyzed the results. QL and XL provided advice and technical assistance. YL wrote the paper. JD

provided critical comments and revised the manuscript. All authors have contributed to and approved the final manuscript.

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Data availability Datasets for this study is available from public databases and has been described in detail in the Methods section of the manuscript. Any team interested in our research could contact us to obtain the complete analysis code.

Declarations

Conflict of interest The authors declared they are no known competing financial interests.

Ethics approval Not applicable.

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