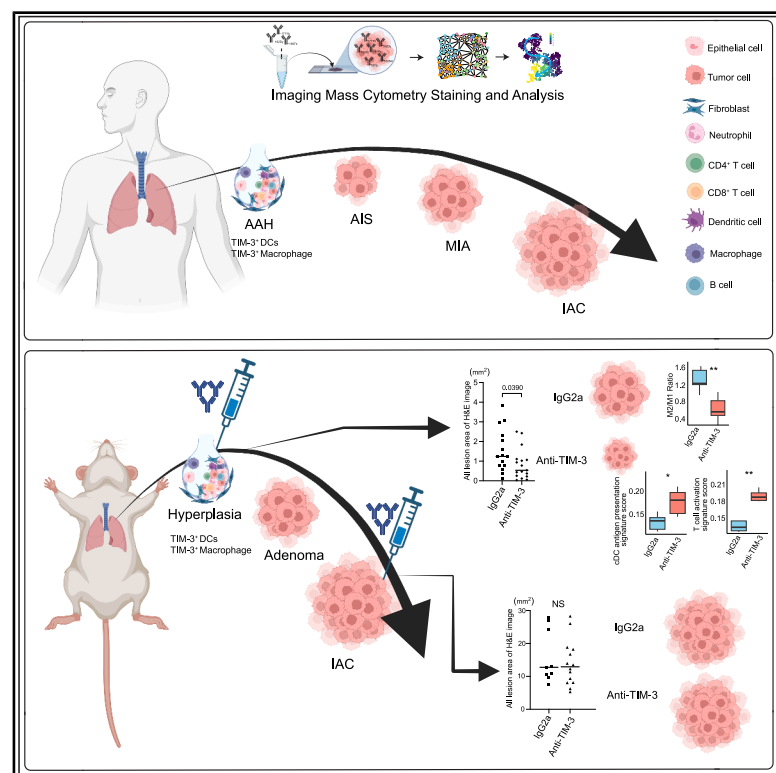


Cancer Cell

Spatial and multiomics analysis of human and mouse lung adenocarcinoma precursors reveals TIM-3 as a putative target for precancer interception

Graphical abstract



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In brief

Zhu et al. reveal coordinated interplay between innate and adaptive immunity in the transition from precancer to invasive lung cancer. TIM-3 upregulation peaks at precancer stage and its blockade enhances anti-tumor immunity while inhibiting lung precancer progression, suggesting TIM-3 as a potential target for lung precancer interception.

Highlights

- Coordinated innate and adaptive immune response shapes lung precancer evolution
- Lung adenocarcinoma precursors have stage-specific tumor microenvironment
- Upregulation of TIM-3 plays a critical role in lung precancer progression
- TIM-3 blockade inhibits progression of precancer but not established lung cancer



Article

Spatial and multiomics analysis of human and mouse lung adenocarcinoma precursors reveals TIM-3 as a putative target for precancer interception

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SUMMARY

How tumor microenvironment shapes lung adenocarcinoma (LUAD) precancer evolution remains poorly understood. Spatial immune profiling of 114 human LUAD and LUAD precursors reveals a progressive increase of adaptive response and a relative decrease of innate immune response as LUAD precursors progress. The immune evasion features align the immune response patterns at various stages. TIM-3-high features are enriched in LUAD precancers, which decrease in later stages. Furthermore, single-cell RNA sequencing (scRNA-seq) and spatial immune and transcriptomics profiling of LUAD and LUAD precursor specimens from 5 mouse models validate high TIM-3 features in LUAD precancers. *In vivo* TIM-3 blockade at precancer stage, but not at advanced cancer stage, decreases tumor burden. Anti-TIM-3 treatment is associated with enhanced antigen presentation, T cell activation, and increased M1/M2 macrophage ratio. These results highlight the coordination of innate and adaptive immune response/evasion during LUAD precancer evolution and suggest TIM-3 as a potential target for LUAD precancer interception.



INTRODUCTION

Lung cancer remains the leading cause of cancer-related mortality globally,¹ largely due to its frequent diagnosis at late stages, which significantly diminishes prospects for cure. The significance of early diagnosis and intervention cannot be overstated, where low dose CT-guided screening has significantly reduced lung cancer-related mortality.²

Understanding the molecular mechanisms underlying early lung carcinogenesis is essential for precise screening, diagnosis, prevention, and treatment. Yet, investigating early carcinogenesis poses significant challenges, particularly in the context of lung adenocarcinoma (LUAD), the most prevalent subtype of lung cancer.³ This challenge primarily stems from the scarcity of LUAD precursor specimens, as surgical resection is not the standard approach for managing LUAD precursors.

Among LUAD precursors, atypical adenomatous hyperplasia (AAH) is the only recognized precancer of LUAD, with the potential to progress into preinvasive adenocarcinoma *in situ* (AIS), minimally invasive adenocarcinoma (MIA), and eventually frankly invasive LUAD (IAC).⁴ These LUAD precursors frequently present as ground glass opacity (GGO)-predominant pulmonary nodules.^{5,6} Obtaining sufficient cells for diagnosis through biopsy of these nodules is often challenging, and surgical resection is not often offered. Consequently, the scarcity of LUAD precursor specimens has impeded our understanding of the molecular features of these precursor lesions.

Our previous studies have revealed a gradual escalation in the genomic and methylation complexity as AAH progresses toward AIS, MIA, and invasive LUAD.^{7–10} Moreover, we have noted a less active and more tightly regulated immune repertoire in later-stage lesions, suggesting ongoing “immunoediting” during neoplastic progression.^{9–11} More recently, studies using high-resolution single-cell RNA sequencing (scRNA-seq) technology have further revealed distinct molecular signatures in LUAD precursors.¹²

The tumor microenvironment (TME) plays a pivotal role in shaping cancer evolution,^{13,14} and the cellular organization within TME dictates its functionality.^{15–22} Although the aforementioned studies have greatly improved our understanding of early LUAD carcinogenesis, they have exclusively utilized next-generation sequencing, which lacks the critical spatial information of TME; or were based on limited immune panels with low resolution to pinpoint TME subsets that could potentially impact early LUAD carcinogenesis. Therefore, the multicellular composition, cell-cell interactions, spatial distribution, and functional dynamics of TME components in LUAD precursors, as well as their interactions with premalignant/malignant epithelial cells, remain largely unexplored. To fill this void, we harnessed the power of imaging mass cytometry (IMC) to acquire single-cell spatial data from human LUAD precursors of various stages and characterized the spatial architecture of the TME across LUAD precursors of different stages. Furthermore, we conducted IMC, scRNA-seq, and spatial transcriptomics profiling of LUAD precursors from five murine LUAD models to validate these findings and performed *in vivo* assays to test novel targets for lung pre-cancer interception.

RESULTS

LUAD precursor progression is associated with the coordination between innate and adaptive immunity

We designed a 34-plex antibody panel and generated spatial single cell TME profiles from a total of 1,618 regions-of-interest (ROIs), derived from 114 LUAD and LUAD precursors (40 AAH, 22 AIS, 18 MIA, and 34 IAC) as well as paired normal lung tissues (Figures 1A, 1B, S1A and S1B, Table S1). In total, we identified 4,828,879 single cells that were classified into 14 major lineages of four cell types: epithelial, lymphoid, myeloid, and stromal cells using an unsupervised clustering approach (Figures 1C–1E, S1C, S1D, S2A and S2B). Notably, the infiltration of major immune cells was not associated with age, gender, or smoking status in this cohort (Table S1), although this may be attributed to the limited sample size within each histologic stage.

Overall, later-stage lesions had higher cell density (number of cells per mm²) across various cell types (Figures 1F and 1G), while the proportions of certain cell types varied substantially across stages (Figure S3). In particular, there was a progressive increase in the proportion of adaptive immune cells (CD4⁺ T cells, CD8⁺ T cells, B cells, and regulatory T cells (Tregs)), accompanied by a progressive decrease in the proportion of innate immune cells (macrophages, neutrophils, monocytes, myeloid-derived suppressor cells (MDSC), natural killer (NK) cells, dendritic cells (DCs)) with progression from normal lung to AAH, AIS, MIA, and IAC (Figures 1H, 1I, and S3A–S3G). Concurrently, the proportions of Ki67⁺PDL1⁺ epithelial cells also increased in later stage lesions (Figures S3H and S3I) suggesting concurrent increases in proliferation and adaptive immune evasion with neoplastic progression.

Early LUAD carcinogenesis is accompanied by macrophage polarization and the functional differentiation of T lymphocytes

We next delved into the details of major cell subsets and their states in LUAD precursors. We first looked into macrophages, the most prevalent immune cell population in the TME (Figures 1E and S3C). The cell density of macrophages progressively increased from AAH to AIS, MIA, and IAC, while the proportion did not show clear trend (Figures S3F and S3G). Interestingly, while the density of CD163⁺ M2 macrophages increased with neoplastic progression, the density of CD163[−] M1 macrophages increased from normal to AAH and AIS but subsequently decreased in MIA and IAC (Figures S3J and S3K), which led to higher M2/M1 ratios in later stages (Figure S3L). Given the anti-tumor functions of M1 and the protumor functions of M2 macrophages,^{23–25} the increased M2/M1 ratio in later stages indicates advancing immunosuppression associated with macrophage polarization during LUAD precursor progression.

Within T lymphocytes that play central roles in anti-tumor immunity, CD4⁺ T cells were classified into naive, memory, proliferating, exhausted CD4⁺ T cells and Tregs. The densities of all five CD4⁺ T cell subtypes increased with tumor progression from AAH to AIS, MIA, and IAC (Figure S3I) consistent with the overall higher infiltration of adaptive immune cells in later stage lesions, as described previously. At the proportion level, the proportions

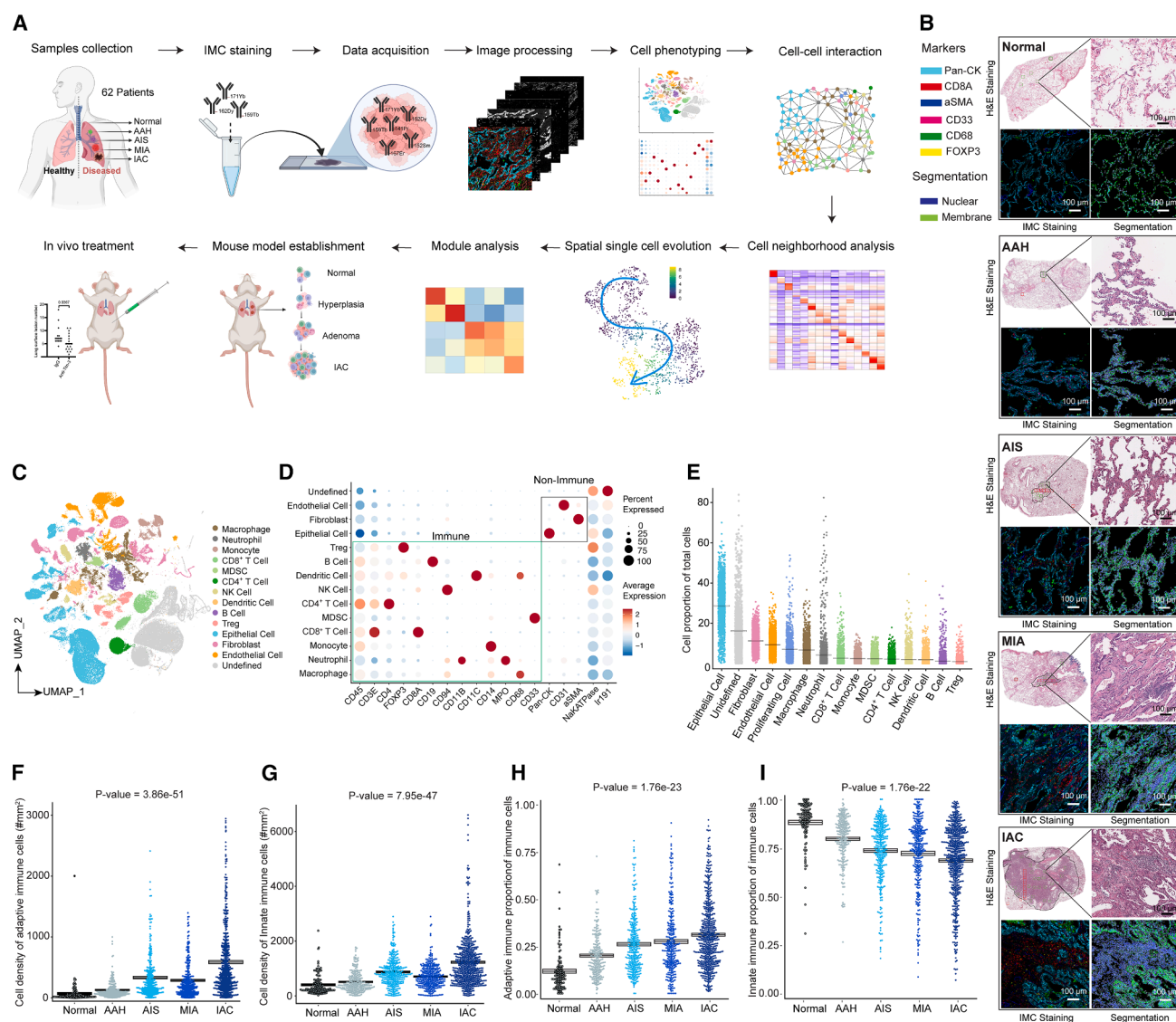


Figure 1. Single-cell imaging mass cytometry reveals the spatial landscape of normal, AAH, AIS, MIA and IAC stages

(A) Schematic illustrating the workflow, including samples collection, IMC staining, data acquisition, imaging processing, cell phenotyping, cell-cell interaction, cell neighborhood analysis, spatial single-cell evolution and module analysis across different human histological subgroups, and mouse models establishment, *in vivo* treatment on animal models. Images were created with BioRender.

(B) Representative images of ROIs selection, IMC staining, and cell segmentation from normal, AAH, AIS, MIA, and IAC. Scale bars, 100 μ m. Each color represents one marker staining for the IMC stained images. Cell segmentations were performed by using the nuclear and membrane markers.

(C) Uniform manifold approximation and projection (UMAP) plot showing all the 15 major cell types of dictionary and query. Cells are colored by their cell-types annotation according to the legend on the right.

(D) Dot plot visualization of each cell type in lung IMC data, the size of the dot encodes the percentage of cells within a cell type, and the color encodes the average expression level.

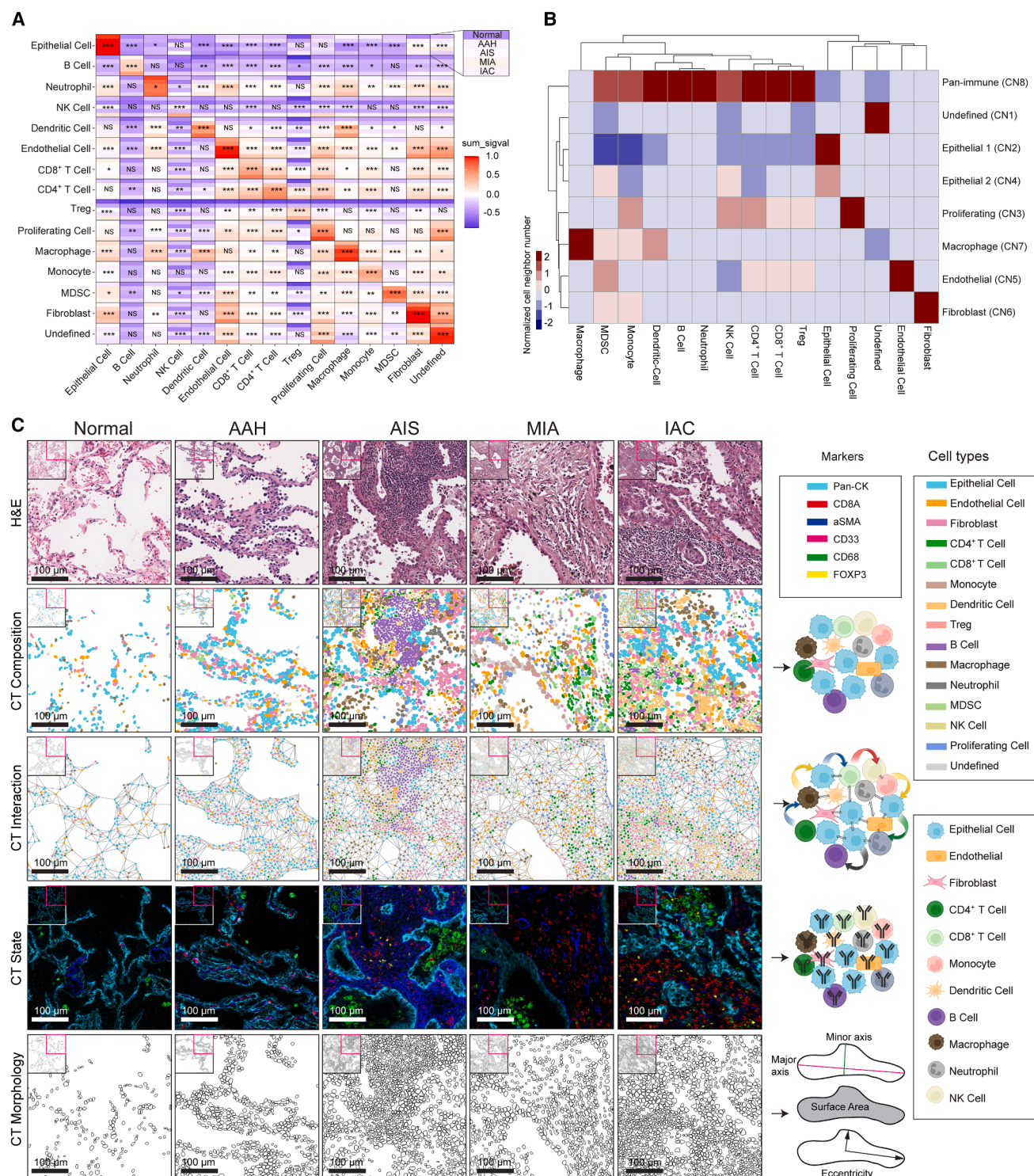
(E) Prevalence of 15 cell types across 114 lesions at different stage of LUAD as a proportion of total cells.

(F and G) The cell density (number of cells/mm²) of adaptive (F) and innate (G) immune cells across normal, AAH, AIS, MIA, and IAC (normal = 116 ROIs, AAH = 226 ROIs, AIS = 313 ROIs, MIA = 308 ROIs, IAC = 561 ROIs). The middle line in the figures denotes the median. Kruskal-Wallis H test for *p* value.

(H and I) The proportion of adaptive (H) and innate (I) immune cells of all immune cells across different histological stages (normal = 116 ROIs, AAH = 226 ROIs, AIS = 313 ROIs, MIA = 308 ROIs, IAC = 561 ROIs). The middle line in the figures denotes the median. Kruskal-Wallis H test for *p* value. See also [Figures S1–S3](#) and [Table S1](#).

of all CD4⁺ T cell subsets increased from AAH to IAC with the exception of naive CD4⁺ T cells, which decreased in late stages ([Figure S3H](#)). Similar to CD4⁺ T cell subsets, the cell density of all five CD8⁺ T cell subsets (naive, memory, proliferating, ex-

hausted, and cytotoxic CD8⁺ T cells) increased with tumor progression ([Figure S3I](#)) and the proportion of all CD8⁺ T cell subsets increased in later stage lesions with the exception of naive CD8⁺ T cells, which decreased at later stages ([Figure S3H](#)). These



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results suggest an ongoing differentiation of naive T lymphocytes into different functional T cell subsets upon antigen exposure and with LUAD precursor progression.

Colocalization of epithelial cells with fibroblasts, Tregs, and macrophages increases from lung precancer to invasive LUAD

To better understand the inter-cellular communications in the TME during early LUAD carcinogenesis, we quantified cell colocalization²⁶ to infer cell-cell “interactions” or “avoidance” between major cell types.¹⁵ Epithelial cells tended to colocalize with other epithelial cells, consistent with “homotypic interactions”-colocalization with the same cell types (Figure 2A). Similar patterns were reported in invasive lung and breast cancers,^{15,19} suggesting that malignant and premalignant cells may communicate with each other to modify their TME and create a protumor niche that supports their growth.

Among the heterotypic interactions-colocalization between different cell types, interactions between epithelial cells and fibroblasts were the strongest, in line with reports that fibroblasts can form a protective fibrotic shield against immune attack.²⁷ Importantly, these heterotypic interactions increased in later-stage lesions (Figure 2A) indicating increasing stromal support for tumor cell growth within TME with neoplastic evolution. Overall, homotypic interactions among major immune cells increased along with neoplastic progression (Figure 2A) indicating a spatially coordinated immune response among the same cell types in the TME during neoplastic evolution. The interaction between Tregs and epithelial cells shifted from avoidance in normal lung tissue to interaction at IAC stage (Figure 2A), suggesting increasing immune regulation toward an immunosuppressive TME in later stage diseases.

Heterotypic interactions between epithelial cells and macrophages also increased as LUAD precursors progressed. The interactions between epithelial cells and M1 versus M2 macrophages were different (Figures 2A and S4A). Overall, M2 exhibited higher heterotypic interactions with epithelial cells than M1 across different histologic stages, especially in the later stages (Figure S4A) in line with their protumor functions. Additionally, the heterotypic interaction between M2 and DCs also increased in later-stage LUAD precursors (Figure S4A) indicating increasing negative control of anti-tumor immunity along with neoplastic evolution, as M2 macrophages can suppress the antigen presenting capabilities of DCs, subsequently impairing CD8⁺ T cell responses.²⁸

Cellular neighborhood analysis highlights the recruitment and organization of immune community during early LUAD carcinogenesis

Next, we defined 8 cellular neighborhoods (CNs) based on the ten nearest spatial neighbors for each cell^{15,17,20} (Figure 2B) to further depict the multicellular TME architecture of LUAD precursors. Notably, the density of immune cell-enriched CN8 (enriched for Tregs, CD8⁺, CD4⁺ T cells, NK cells, neutrophils, B

cells, DCs, monocytes, and MDSC) progressively increased in later stage lesions, once again suggesting an increasing host immune pressure by recruiting various immune cells to impede cancer progression (Figure S4B). Interestingly, however, the density of macrophage-enriched CN7 showed an early increase followed by a slight decrease as LUAD progresses (Figure S4B). To validate this, we applied Hopkins statistic²⁹ to quantify clustering tendency of macrophages within each lesion independent of CNs, which also exhibited an early increase followed by a slight decrease in later stages (Figure S4C). Taken together, these results suggest an active organization of macrophages during very early phases of LUAD carcinogenesis.

LUAD precursors of different stages have distinct multicellular TME modules

To comprehensively elucidate the interplay among cell composition, cellular organization, and multicellular TME structure, the aforementioned derived cell types (CT) and CNs were categorized into four categories: (1) CT/CN composition; (2) CT/CN interaction; (3) CT/CN states; (4) CT/CN morphology (Figure 2C, Table S2). This comprehensive analysis resulted in a digitized TME atlas comprising 818 parameters (Figures S4D and S4E) of each specimen. Monocle 3³⁰ analysis confirmed an overall trajectory commencing with normal lung tissue and progressing through AAH, AIS, MIA, culminating in IAC stage (Figures 3A, 3B, S5A, and S5B).

Next, using feature co-expression analysis, we established five distinct multicellular spatial modules enriched in each histologic stage. As shown in Figures 3C and S5C and Table S3, normal tissue was enriched for module 1 (endothelial parameters enriched module), reflecting the role of endothelial cells (ECs) in alveolar gas exchange.³¹ The AAH stage was enriched for module 2 (TIM-3 enriched module), characterized by cell state features, especially features with high expression of TIM-3 on MDSC, macrophages, CD4⁺, and CD8⁺ T cells, among others. As TIM-3 is a crucial checkpoint regulating both innate and adaptive immune response,^{32,33} these observations highlight the coordination of innate and adaptive immunity at this stage and indicate the important role of TIM-3 at LUAD precancer stage. AIS stage was enriched for module 3 (NK-Treg enriched module). Tregs exhibited high heterotypic interactions with NK cells, cytotoxic T cells, MDSCs, DCs, and macrophages underscoring the important role of Tregs at the AIS stage. MIA stage was enriched for module 4 (B cell enriched module). Notably, homotypic interactions of B cells were highly enriched, and heterotypic interactions of B cells with other immune cells, epithelial, and stromal cells were also prevalent at MIA stage. Finally, IAC was characterized by module 5 (KI-67 parameters enriched module) highlighting the significance of uncontrolled cell proliferation in invasive LUAD. In addition, features associated with immune checkpoint molecules and costimulatory/co-inhibitory immunoregulatory protein B7-H3 (CD276) were also highly enriched at the IAC stage compared to other stages (Figure S5D).

(C) Representative visualizations of spatial features, including cell composition (proportion and density; each color represents a different cell type); cell-cell interactions (each colored dot represents a cell with connections indicating interactions); cell state features (each color represents a marker expression with brightness indicating expression intensity); cell morphology features (major axis length, minor axis length, surface area, and eccentricity). Scale bars, 100 μ m. Images were created with BioRender. See also Figure S4 and Table S2.

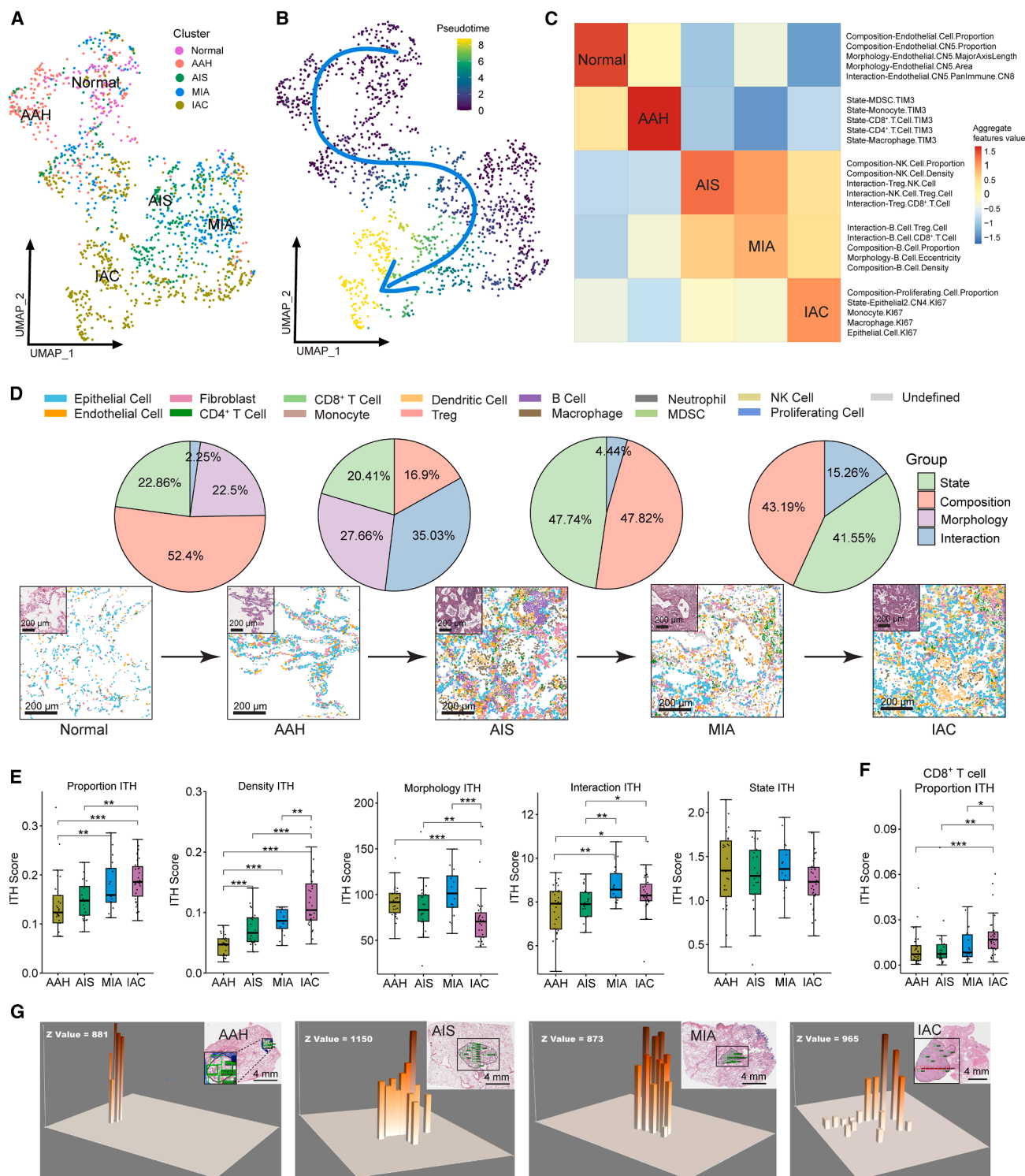


Figure 3. Transitions from normal lung to AAH, AIS, MIA, and IAC are marked by changes in the multicellular TME modules

(A and B) UMAP view of 1,618 ROIs and displaying the distribution of inferred pseudotime across different stages during early LUAD carcinogenesis. Each dot in the UMAP represents one ROI. The arrow represents the pseudotime trajectory path.

(C) Heatmap of the distinguishing feature prevalence in normal, AAH, AIS, MIA, and IAC samples. Co-expression analysis identified five groups of distinct feature-enrichment modules in the tissues states, including those highest in normal tissue and low in other stages (module 1: normal enriched), those highest in AAH tissues (module 2: AAH enriched), those highest in AIS tissues (module 3: AIS enriched), those highest in MIA tissues (module 4: MIA enriched), and those highest in IAC tissues (module 5: ADC enriched). Representative features were showed for each module.

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Different TME organization features constituted the transitions from normal to AAH, AIS, MIA, and invasive LUAD

To comprehensively quantify the molecular and immune features that shape early LUAD carcinogenesis, we compared how these features (CT/CN state, composition, morphology, and interaction) changed in the transition from normal lung to AAH, AIS, MIA, and IAC. Our analysis revealed that during the transition from normal lung to AAH, Composition features contributed 52.4% of the differences, State features accounted for 22.86%, and morphology 22.5%. These results indicate that alterations in cell proportion, density, state, and morphology were the most significant changes during precancer initiation. Interaction features only accounted for 2.25%, suggesting minimal change in cell communication at these two stages (Figure 3D, Table S4). As AAH progressed to AIS, interaction features increased to 35.03% and became predominant, indicating increased cell communication at this transition. This was followed by morphology features that increased to 27.66%, aligning with histological variations. This transition was associated with smaller changes in cell proportion and composition (16%). At the transition from AIS to MIA, state features and composition features again became predominant, making up almost all the contributions at 47.74% and 47.82%, respectively. And finally, from MIA to IAC, state features (41.55%) and composition features (43.19%) continued to play major roles, while Interaction features contributed for 15.26%. Notably, morphology features contributed significantly from normal lung to AAH, AIS, and MIA, but their contribution diminished as LUAD progressed to IAC.

LUAD precancer progression is associated with progressive increase in TME intra-tumor heterogeneity

Tumor heterogeneity is a fundamental characteristic of cancer with significant implications for cancer diagnosis, treatment, and prognosis.^{13,34} Our recent work revealed that intratumoral heterogeneity (ITH) emerges at precancer stages.⁷ However, previous studies have primarily focused on genetic and epigenetic heterogeneity,^{13,35–38} and immune ITH analysis has been limited by small panels of immune markers. Therefore, we next evaluated the ITH of multicellular spatial organization across LUAD precursors of different stages. We quantified ITH of cell density, cell proportion, cell morphology, cell state, and cell-cell interaction by the average Euclidean distances of the corresponding attributes across pairwise comparisons of ROIs (Figure 3E). Overall cell morphology ITH did not differ significantly

among AAH, AIS, and MIA but significantly decreased at IAC stage (Figure 3E), suggesting convergence of cell morphology at invasive LUAD stage. On the other hand, cell density ITH and proportion ITH increased with tumor progression for overall immune infiltration (Figure 3E) and for individual immune cell types (Figures 3E–3G) indicating a more complex and heterogeneous TME, which may further impair anti-tumor immunity at later stages.

TIM-3 expression increases at early phases of carcinogenesis in human LUAD and mouse models

As one important goal of our study was to identify novel targets for LUAD precancer interception, we delved into TIM-3 because: (1) TIM-3-high features are enriched at AAH stage (Figures 3C, 4A, 4B and S4E, Table S3) making it an appealing target for precancer interception; and (2) multiple anti-TIM-3 agents have shown good safety profiles in clinical trials,^{39–43} which are critical for interception/prevention studies.

In line with high infiltration of innate immune cells at AAH stage, the highest TIM-3 expression was observed in various innate immune cells including DCs, macrophages, and MDSCs (Figure 4C, Table S3). Furthermore, as shown in Figures S5E–S5G, the high TIM-3 expression at AAH stage is not only driven by the expression of TIM-3 in various cells, but also by the proportion of TIM-3⁺ cells. Moreover, despite of overall higher cell density in later stage lesions across different cell types, the absolute numbers of TIM-3⁺ macrophages, DCs and MDSCs peaked at AAH or AIS stage highlighting its crucial role during early phases of LUAD carcinogenesis.

To further examine the role of TIM-3 in LUAD precursor evolution and prepare for functional validation, we developed five LUAD precancer models with different genetic backgrounds and etiologies including 4 genetically engineered mouse models (GEMMs) (129S4 K [129S4/Sv-Kras^{G12D/+}], C57BL6 K [C57BL6-Kras^{G12D/+}], C57BL6 KP [C57BL6-Kras^{G12D/+}p53^{R172H/+}], and C57BL6 KLL [C57BL6-Kras^{G12D/+}Lkb1^{flox/flox}]) and a carcinogen-induced LUAD precancer model (urethane-induced LUAD in 129S4/Sv wild-type mice) (Figures S6A and S6B, Table S5). Tissues histologically resembling human LUAD and its precursors, including normal, hyperplasia (resembling human AAH), adenoma (resembling human AIS and MIA), and adenocarcinoma (resembling human IAC) were defined by experienced thoracic pathologists⁴⁴ (Figure S6B).

We designed a 39-plex IMC antibody panel (Figures S6C–S6E) to profile TME of mouse LUAD precursors of different stages. In total, we generated 3,263,713 single cell profiles from 589 LUAD

(D) Feature contribution analysis across five histological stages. Differentially expressed (DE) features were performed by comparing the pairs of groups using Wilcoxon rank-sum test. A cutoff was set such that DE analysis was performed only on features that associates with at least 25% of samples in both the compared stages. Feature importance was then computed using the returned adjusted *p* values. Each color in ROI represents one kind of cell. Green color in pie chat represents CT&CN states features; red color in pie chat represents CT&CN composition features; purple color in pie chat represents CT&CN morphology features; blue color in pie chat represents CT&CN interaction features, respectively. Scale bars, 200 μ m.

(E) Overall CT&CN proportion, CT&CN density, CT&CN morphology, CT&CN interaction, and CT&CN states intratumor heterogeneity across different LUAD stages (40 AAH, 22 AIS, 18 MIA, and 34 IAC). The center line in the box shows the median. The bottom and top of the box show the 25th and 75th percentiles. For each feature, the ITH was derived from the average Euclidean distances of the corresponding attributes across pairwise comparisons of ROIs, two tailed tests for *p* value (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

(F) CD8⁺ T cell Proportion ITH between different LUAD stages (40 AAH, 22 AIS, 18 MIA, and 34 IAC). The center line in the box shows the median. The bottom and top of the box show the 25th and 75th percentiles. Two tailed tests for *p* value (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

(G) Representative cases showing CD8⁺ T cell proportion ITH from different LUAD stages. Each column in the figure represents one ROI location corresponding to the ROIs selection in H&E images. The normalized Z value means the CD8⁺ T cell proportion of each ROI. See also Figure S5 and Tables S3 and S4.

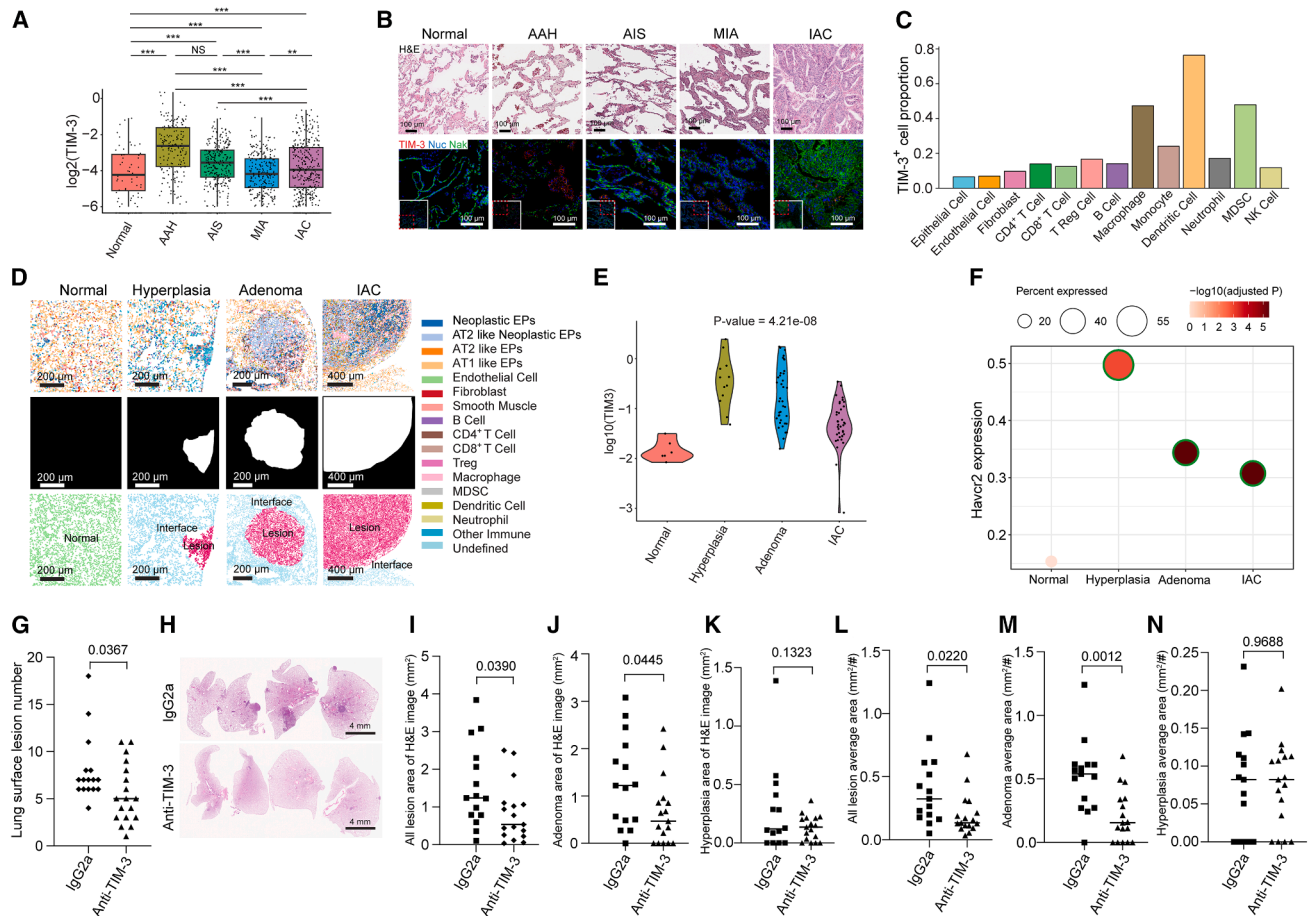


Figure 4. TIM-3 expression patterns in human and mouse models and antibody blockade in mouse model

(A) Relative TIM-3 expression in each lesion (normal, AAH, AIS, MIA, IAC) across different histological stages (normal = 116 ROIs, AAH = 226 ROIs, AIS = 313 ROIs, MIA = 308 ROIs, IAC = 561 ROIs). The center line in the box shows the median. The bottom and top of the box show the 25th and 75th percentiles. Wilcoxon tests for p value. p value adjustment method: Bonferroni (NS: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

(B) Representative images to show the TIM-3 expression across different histological stages. Scale bars, 100 μm . Blue color represents the Ir191 nuclear staining; green color represents the NaKATPase membrane staining; and red color represents TIM-3 staining, respectively.

(C) Overall TIM-3⁺ cell proportion of all 13 major cell types in all human LUAD precursor specimens of various stages.

(D) Top panel: representative images of different pathological lesions showing the spatial localization of 17 major cell types (each color represents one cell type). Middle panel: representative images of segmentation mask showing the lesion area and non-lesion area. Bottom panel: highlighted regions of normal, lesion interface and lesion area by different colors (light green for normal; light blue for lesion interface and light red for lesion). The data are from 129S4 U model.

(E) TIM-3 mean expression across different stages including normal ($n = 6$), hyperplasia ($n = 12$), adenoma ($n = 38$), and adenocarcinoma ($n = 39$) of 129S4 U model. Kruskal-Wallis H test was used to assess whether TIM-3 expression was significantly different among different stages.

(F) *Hacr2* expression across different histological stages of 129S4 U model. The height of each dot on the y axis represents its mean expression value. Color depth represents the adjusted p value (FDR), which is calculated by the two-tailed Wilcoxon rank-sum test with normal as the reference. The expression value and p value here only compare “expressed spots” to avoid that the difference in expression comes from the proportion of expressed spots. The size of dot represents the percentage of spots expressing the gene. The green circle outside dot indicates that it is significant (FDR < 0.01).

(G) Disease burden by the number of lesions on lung surface. Mice from 129S4 U model were sacrificed after 10 weeks of treatment with IgG2a ($n = 16$) or anti-TIM-3 ($n = 18$) antibody. The number of lesions on lung surface was calculated. The middle line in the figures denotes the median. Two tailed tests for p value.

(H) Representative H&E images from IgG2a and anti-TIM-3 antibody treatment groups to show the lesions and lung tissue staining. Scale bars, 4 mm. The data are from 129S4 U model.

(I–K) The disease burden (total areas in mm^2) for all lesions (I), adenomas (J), and hyperplasia (K) from each mouse based on pathologic assessment on H&E images. Mice from 129S4 U model were sacrificed after 10 weeks of treatment with IgG2a ($n = 15$) or anti-TIM-3 ($n = 17$) antibody. The middle line in the figures denotes the median. Two tailed tests for p value.

(L–N) The average lesion size (area in mm^2) of all lesions (L), adenomas (M), and hyperplasia (N) based on pathologic assessment on H&E images from 129S4 U model after treatment with IgG2a ($n = 15$) or anti-TIM-3 ($n = 17$) for 10 weeks. The middle line in the figures denotes the median. Two tailed tests for p value. See also Figures S4–S10 and Tables S3, S5 and S6.

precursors of various stages and paired normal lung tissues (268 normal, 73 hyperplasia, 120 adenoma, and 128 adenocarcinoma) from aforementioned 5 mouse models (Figures 4D,

S6C, and S6D, Table S5). These cells were then classified into 16 major cell types (Figures S7A–S7D). Consistent with results from human specimens, TIM-3 expression peaked at

hyperplasia or adenoma stages in all 5 models (Figures 4E, S7E–S7K). Importantly, TIM-3 expression was also enriched in DCs, macrophages, and MDSCs and peaked at hyperplasia stage in 4 of 5 models (Figures S7G–S7K), similar to human specimens (Figure S5E).

To orthogonally validate these findings, we performed Visium spatial transcriptomics profiling of 484 LUAD precursors and paired normal lung tissues (48 normal, 277 hyperplasia, 133 adenoma, and 26 adenocarcinoma), which resulted in a total of 91,083 ST spots (Figures S8A–S8E, Table S6). *Havcr2* (encoding for TIM-3) expression peaked at hyperplasia or adenoma stages across all 5 models and the difference was significant in 3 of 5 models (Figures 4F, S9A–S9E). Taken together, these results suggest that upregulation of TIM-3 may be an important immune evasion mechanism during early LUAD carcinogenesis, which is conserved in human and mouse LUADs of various genetic background and etiologies.

Ceacam1 may mediate immune evasion at LUAD precancer stage through TIM-3 binding

Further, we analyzed spatial transcriptomics and scRNA-seq data to identify potential ligands interacting with TIM-3 that mediate immune evasion during early LUAD carcinogenesis. We used the urethane-induced S129S4 model because it exhibited the highest level of TIM-3 expression among these five models; TIM-3 expression peaked at hyperplasia stage (Figures S7E and S7F); and the high TIM-3 expression was primarily enriched in DCs, macrophages, and MDSCs (Figures S7G–S7K) consistent with human specimens (Figures 4A–4F, S5E). Using Visium spatial transcriptomics, we examined the expression of genes encoding TIM-3 ligands, including *Ceacam1*, *Hmgb1*, *Lgals9*, and *Ptdser*.⁴⁵ Among these, *Ceacam1* emerged as the only gene exhibiting a similar expression pattern to *Havcr2* (encoding TIM-3), with both genes showing low co-expression in normal lung but significantly increased expression at hyperplasia stage (Figure S9F).

Given the limitations of low-resolution, spot-level data from Visium, we further analyzed scRNA-seq data from the same model, with samples collected at different time points during early LUAD carcinogenesis (Figures S9G and S9H). Consistent with IMC data (Figures S7G–S7K), *Havcr2* expression was the highest in myeloid cells, including DCs, macrophages, monocytes, and neutrophils (Figure S9I). Ligand-receptor analysis of the scRNA-seq data confirmed that *Ceacam1* was upregulated at early time points, primarily in epithelial cells. Furthermore, a significant interaction probability was detected between *Ceacam1*-expressing epithelial cells and *Havcr2*-expressing myeloid cells (Figure S9J). Collectively, these findings suggest a prominent *Ceacam1*–*Havcr2* interaction between epithelial cells and innate immune cells during early LUAD carcinogenesis.

TIM-3 blockade at the precancer stage attenuates tumor progression

Next, we examined whether TIM-3 blockade intercepts LUAD precancer progression. For the *in vivo* experiments, we used the same aforementioned S129S4 model. Specifically, 4 weeks after induction when hyperplasia lesions emerge (Figure S6B), mice were randomized to 2 treatment arms: IgG2a isotype con-

trol and anti-TIM-3 IgG monoclonal antibody (Figure S10A). No severe adverse effects were observed from these treatments. 10 weeks after treatment (14 weeks after induction), mice were sacrificed for interception efficacy assessment.

By gross examination, the numbers of visible lesions on the lung surfaces were significantly lower in mice treated with anti-TIM-3 than those receiving IgG2a controls (Figures 4G and S10B). Moreover, by histologic assessment (Figure 4H), the overall disease burden (the areas of lesions of all stages) were significantly lower in the anti-TIM-3 group than in the IgG2a group (Figure 4I). Furthermore, we observed that the disease burden was significantly lower in the anti-TIM-3 group than in the IgG2a group for adenoma burden (Figure 4J) but not for hyperplasia burden (Figure 4K). Finally, we looked into the lesion size (total disease area divided by the number of lesions), and found that anti-TIM-3 was associated with smaller adenoma (but not smaller hyperplasia) lesions than IgG2a (Figures 4L–4N). These findings further support the role of TIM-3 in immune evasion during early LUAD carcinogenesis and indicate the potential of TIM-3 blockade for LUAD precancer interception.

Importantly, TIM-3 blockade administered at a later stage in the same model, when invasive LUAD was fully developed (Figures S6A and S6B), did not significantly reduce the disease burden compared to IgG2a controls (Figures S10C–S10E). These findings suggest that the efficacy of anti-TIM-3 treatment may be stage-dependent and TIM-3 blockade may give greater potential for intercepting LUAD precursors than treating invasive LUAD.

TIM-3 blockade enhances antigen presentation and immune-mediated cell killing

To investigate the mechanisms of action of TIM-3 blockade in LUAD precancer interception, we conducted Xenium 5K single-cell spatial transcriptomics profiling on samples from mouse specimens treated with either anti-TIM-3 or IgG2a control (Figure 5A). This analysis yielded a total of 1,407,893 spatially resolved single cells. Integration with scRNA-seq data from the same model (Figures S9G and S9H) allowed us to identify 12 major cell types (Figures 5B and 5C).

In comparison to the IgG2a-treated mice, the anti-TIM-3-treated group exhibited a significant reduction in the proportion of epithelial cells (Figures S10F and S10G), in line with a decrease in tumor burden by TIM-3 blockade. Among the immune cells, macrophages constituted the largest immune subset, consistent with findings from IMC profiling. Anti-TIM-3 treatment significantly reduced macrophage populations (Figures 5D, S10F and S10G). These findings align with prior reports that ablation of macrophages, particularly senescent macrophages, enhances immunosurveillance and reduces tumor burden in LUAD models.^{46–48} Interestingly, there was a more pronounced decrease in protumor M2 macrophages than anti-tumor M1 macrophages (Figure 5E) leading to a significantly lower M2/M1 ratio in the anti-TIM-3-treated group (Figure 5F).

Conversely, TIM-3 blockade was associated with a significantly higher proportion of conventional dendritic cells (cDCs)—a critical immune subset for antigen presentation—compared to the IgG2a control group (Figure 5D). Further analysis revealed that cDCs from anti-TIM-3-treated mice

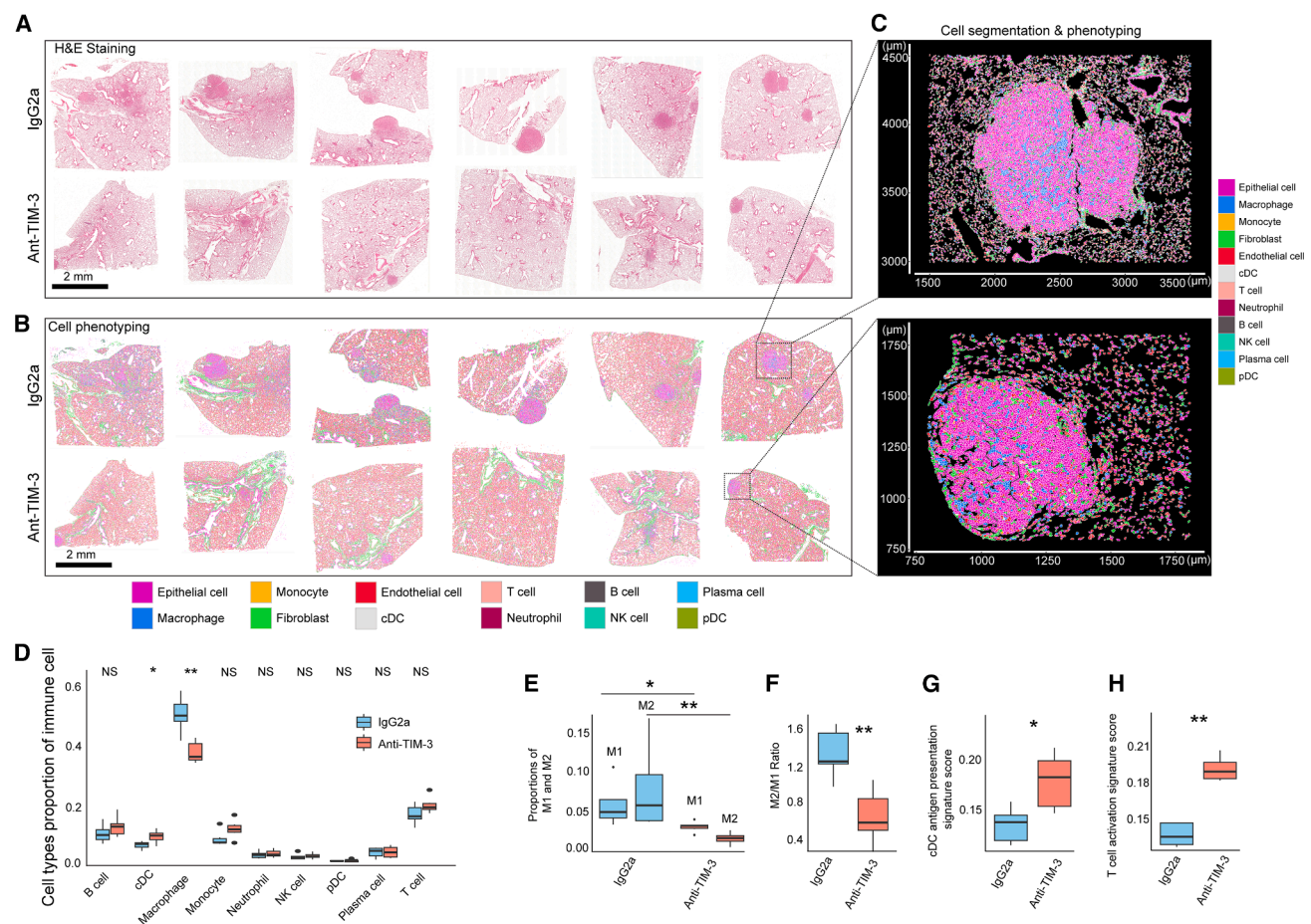


Figure 5. Spatial single cell transcriptomics profiling by Xenium 5k of mouse lung specimens after treatment by anti-TIM-3 or IgG2a controls

(A) H&E images of tissues from mice of 129S4 U model after treatment with anti-TIM-3 ($n = 6$) or IgG2a ($n = 6$) controls. Scale bars, 2 mm.

(B) Cell phenotyping map of tissues from IgG2a ($n = 6$) and anti-TIM-3 ($n = 6$) treatment groups. Each color represents one cell type. Scale bars, 2 mm.

(C) Representative images showing the cell segmentation and cell phenotypes in IgG2a and anti-TIM-3 treatment groups. The numbers on x axis and y axis represent spatial coordinates in microns and the size of the tumor respectively.

(D) The proportions of major immune cell types as of the total immune cell from IgG2a (blue, $n = 6$) or anti-TIM-3 (red, $n = 6$) treatment groups. The center line in the box shows the median. The bottom and top of the box show the 25th and 75th percentiles. Student's t test for p value (NS: not significant, $*p < 0.05$, $**p < 0.01$).

(E) The proportion of M1 and M2 macrophages from IgG2a (blue, $n = 6$) or anti-TIM-3 (red, $n = 6$) treatment groups. The center line in the box shows the median. The bottom and top of the box show the 25th and 75th percentiles. Wilcoxon test for p value ($*p < 0.05$, $**p < 0.01$).

(F) The ratio of M2/M1 macrophages from IgG2a (blue, $n = 6$) or anti-TIM-3 (red, $n = 6$) treatment groups. The center line in the box shows the median. The bottom and top of the box show the 25th and 75th percentiles. Wilcoxon test for p value ($**p < 0.01$).

(G) The antigen presentation signature score in cDC from mice treated with IgG2a (blue, $n = 6$) or anti-TIM-3 (red, $n = 6$). The center line in the box shows the median. The bottom and top of the box show the 25th and 75th percentiles. Wilcoxon test for p value ($*p < 0.05$).

(H) The T cell activation signature score from mice treated with IgG2a (blue, $n = 6$) or anti-TIM-3 (red, $n = 6$). The center line in the box shows the median. The bottom and top of the box show the 25th and 75th percentiles. Wilcoxon test for p value ($**p < 0.01$). See also [Figures S9](#) and [S10](#).

exhibited significantly higher antigen presentation scores relative to those from IgG2a group ([Figure 5G](#)). Moreover, T cells in the anti-TIM-3 treatment group demonstrated significantly higher activation scores than their counterparts in the IgG2a group ([Figure 5H](#)).

Collectively, these findings suggest that TIM-3 blockade may enhance anti-tumor immunity by improving antigen presentation (increased proportion of antigen-presenting cDCs and their enhanced antigen presentation capacity); by strengthening immune-mediated cell killing via T cells (increased T cell activation), and by a favorable shift in the M1/M2 macrophage ratio toward anti-tumor activity.

DISCUSSION

Lung cancer remains the foremost cause of cancer-related fatalities. This underscores the imperative need for the prevention of invasive lung cancers. Active intervention targeting preinvasive cancer precursors, referred to as “interception”,^{49,50} presents a promising avenue for cancer prevention. However, its implementation in LUAD prevention is challenging due to our limited understanding of the complicated interplay between premalignant/malignant cells and anti-tumor immunity during LUAD pre-cancer formation and progression. In this study, we employed high-resolution IMC profiling and characterized the detailed

TME structure and cellular composition at spatial and single cell levels; identified distinctive spatial TME features associated with each histologic stage during LUAD early carcinogenesis; and discovered a promising lung cancer interception target TIM-3 that is undergoing further development.

The scarcity of LUAD precursor specimens has long hindered our understanding of early LUAD carcinogenesis. We herein constructed a comprehensive, multi-compartmental atlas of LUAD precursors. This atlas showcases the complete spectrum of cell phenotypes, states, neighborhoods, and TME architectures of LUAD and its precursors. Our findings unveiled the differential contributions of innate and adaptive immunity at various histologic stages during early LUAD carcinogenesis across cellular, spatial, and multicellular levels. Specifically, we observed evidence of a coordination between innate and adaptive immune responses during the initiation and progression of LUAD precancers. These results indicate that at the onset of LUAD carcinogenesis, non-specific, rapidly acting innate immunity may serve as a primary defense mechanism against cancer initiation. However, as precancer progresses, this defense gradually shifts toward the highly specific and robust adaptive immune response. Accordingly, immune evasion mechanisms vary at different evolutionary stages, corresponding to the immune response patterns at each stage. For instance, at AAH stage, the innate immune response appeared to be the primary contributor to anti-tumor immunity and the adaptive immune response had just begun to emerge. Consequently, the upregulation of TIM-3, which regulates both innate and adaptive immunity,^{32,43,51} serves as a crucial mechanism underlying immune evasion at AAH stage. As precancer progresses from AAH to AIS, MIA, and IAC, adaptive immunity increasingly takes precedence. Concurrently, mechanisms of adaptive immune evasion, such as the upregulation of immune checkpoints like PD-L1 and CTLA-4 become more prominent, while the relative role and contribution of TIM-3 may diminish. These findings suggest that while targeting adaptive immunity may hold potential for immune interception in later-stage IPNs, reprogramming innate immunity may be more effective for intercepting earlier stage LUAD precursors.

These insights have formed the crucial scientific foundation for our immune interception trials Can-Prevent-Lung (testing Canakinumab, an interleukin-1 β antibody for the interception of LUAD precursors, NCT04789681) and IMPRINT-Lung (testing Pembrolizumab, an anti-PD-1 antibody for the interception of LUAD precursors, NCT03634241). The early success of these trials⁵² underscores the promise of immune interception for LUAD prevention. Furthermore, this extensive dataset has facilitated identifying new targets for lung cancer interception and we identified TIM-3 as a putative target. TIM-3 blockade at very early phases of LUAD carcinogenesis significantly reduced disease burden. As multiple anti-TIM-3 agents have been tested in human trials showing good safety profiles,^{40–43} these results suggest that TIM-3 blockade can be a promising avenue for immune interception to prevent LUAD.

From a mechanistic perspective, TIM-3 is a well-known immune checkpoint molecule that regulates both innate and adaptive immunity.^{32,43} In our dataset, TIM-3 is expressed on multiple cell types, with the highest levels on DCs, macrophages, and MDSCs in both human and mouse LUAD precursors. It is well es-

tablished that DCs, as antigen-presenting cells, play a critical role in T cell activation and priming, whereas MDSCs directly inhibit T cell activation.⁵³ TIM-3 engagement has been suggested to decrease antigen presentation.⁵¹ Furthermore, TIM-3 can interact with Gal-9 on MDSCs to promote their expansion and suppress T cell responses.^{54,55} The roles of macrophages in cancer biology are more complex. Macrophages are composed of heterogeneous subsets with distinct phenotype and functions. In LUAD, macrophages have been shown to promote tumor development, particularly following exposure to PM2.5 air pollution.²⁴ The ablation of senescent macrophages enhances immunosurveillance and reduces tumor burden in LUAD models.^{46,48} While M2 macrophages are associated with tumorigenesis and progression,⁵⁶ M1 macrophages can exert anti-tumor effects through direct cytotoxicity⁵⁷ or antibody-dependent cell-mediated cytotoxicity.⁵⁸ Single-cell spatial transcriptomics profiling by Xenium 5K of mouse specimens treated with anti-TIM-3 revealed that TIM-3 blockade is associated with enhanced antigen presentation in DCs, reduced macrophage infiltration, a higher M1/M2 macrophage ratio, and improved T cell activation. Thus, TIM-3 likely exerts its immune evasion functions at LUAD precancer stages by suppressing antigen presentation and impeding the cytotoxic activity of macrophages and T cells. These effects can be reversible with TIM-3 blockade at precancer stage. It is important to note that anti-TIM-3 is much less effective when administered at advanced stages suggesting that therapeutic vulnerability may be dependent on the stage-specific biological features, which should be considered when developing therapeutic strategies.

Limitations of this study

The primary aim of our study was to investigate the evolution of LUAD precursors. Similar to previous studies, our investigation was based on a linear evolutionary model, progressing from normal lung to AAH, AIS, MIA, and eventually IAC. However, it remains uncertain whether all AAH lesions would progress to IAC or whether all LUADs adhere to the linear trajectory from AAH through AIS and MIA to IAC. Our multicellular TME module analysis revealed an overall trajectory from normal lung to AAH, AIS, MIA, and finally IAC. However, we observed significant overlap between stages and clusters. While this may partly reflect the substantial heterogeneity between different lesions of the same stages,^{7,8,59–66} these findings may also indicate alternative branch evolution for certain patients. Unfortunately, this critical question cannot be addressed by the dataset from the current study, because like prior studies on LUAD evolution,^{7,8,11,67–69} our work relied on resected specimens, which provide only a single snapshot of the evolutionary process. Longitudinal specimens by serial biopsies could provide valuable insights into the temporal events and possible alternative branch evolution driving the progression of certain LUAD precursors.

From a technical perspective, while IMC profiling provides deep, granular, and spatial data on TME architecture, the technology has notable limitations. First, as an immunostaining-based imaging technique, the quality of IMC data depends heavily on the availability and reliability of marker proteins. Despite significant advancements in scRNA-seq, the development of protein markers has not kept pace, resulting in a limited repertoire of reliable markers for optimal cell phenotyping, particularly for myeloid

cells.⁷⁰ Second, many protein markers are not uniquely expressed by a single cell type but are instead shared across multiple cell types,⁷¹ further complicating cell classification. Third, variability in signal intensities among marker proteins can hinder data visualization. Finally, challenges in imaging analysis, such as imperfect single-cell segmentation, can lead to contamination from adjacent cells, impacting the accuracy of cell annotation.^{72,73} To mitigate these limitations, we employed a machine learning-based approach for cell annotation that considers the expression of all markers simultaneously, rather than relying on a stepwise, single-marker gating strategy. However, these technical challenges may still result in some degree of contamination among cell types, particularly within myeloid populations in this study and prior studies utilizing IMC.^{19,20,22,74} Reassuringly, the cell annotations derived from IMC closely align with those from scRNA-seq, which included many more markers, providing confidence in our IMC-based phenotyping.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Jianjun Zhang (jzhang20@mdanderson.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All the original data presented in this study are publicly accessible at Synapse (<https://www.synapse.org/Synapse:syn54951674>) including human IMC data (syn61802885), mouse IMC data (syn61811851), mouse Visium spatial transcriptomics data (syn61842020), and mouse Xenium 5K data (syn63942456). The mouse scRNA sequencing data are publicly accessible at GEO (GSE293720). Codes for conducting the imaging mass cytometry, scRNA-seq and spatial transcriptomics analysis are accessible at GitHub (<https://github.com/WuLabMDA/LungIMC>). All software programs used for analyses are publicly available and listed in the [key resources table](#).

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AUTHOR CONTRIBUTIONS

Conceptualization, J. Zhang and B.Z.; methodology, B.Z., P.C., and M.A.; software, P.C. and M.A.; validation, J. Zhang and B.Z.; formal analysis, P.C., M.A., J.-R.L., and H.C.; investigation, B.Z.; resources, J. Fujimoto, B.Z., L.H., and F. R.R.; data curation, B.Z., P.C., and M.A.; writing – original draft, J.Z. and B.Z.; writing – review and editing, all authors.; visualization, B.Z.; supervision, J.W., and J. Zhang; funding acquisition, J. Zhang.

DECLARATION OF INTERESTS

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STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal Anti-human CD45RO (T200/797) antibody	Abcam	Cat# ab212786; RRID: AB_3676102
Rabbit monoclonal Anti-human & mouse aSMA (EPR5368) antibody	Abcam	Cat# ab220795; RRID: AB_3676095
Rabbit monoclonal Anti-human ICOS (D1K2T) antibody	CST	Cat# 89601BF; RRID: AB_2800142
Rabbit monoclonal Anti-human HLA-DR (EPR3692) antibody	Abcam	Cat# ab215985; RRID: AB_2864390
Rabbit monoclonal Anti-human CD68 (EPR20545) antibody	Abcam	Cat# ab227458; RRID: AB_3676097
Rabbit monoclonal Anti-human MPO (EPR20257) antibody	Abcam	Cat# ab221847; RRID: AB_3086778
Rabbit monoclonal Anti-human TIGIT (BLR047F) antibody	Abcam	Cat# ab243903; RRID: AB_2943164
Rabbit monoclonal Anti-human CD11c (EP1347Y) antibody	Abcam	Cat# ab216655; RRID: AB_2864379
Rabbit monoclonal Anti-human CD73 (D7F9ABF) antibody	CST	Cat# 13160BF; RRID: AB_2716625
Rabbit monoclonal Anti-human PD-L1 (SP142) antibody	Standard BioTools	Cat# 3150033D; RRID: AB_3106929
Rabbit monoclonal Anti-human CD163 (BLR087G) antibody	Bethyl	Cat# A700-087; RRID: AB_2891884
Rabbit monoclonal Anti-human Granzyme B (D6E9W) antibody	CST	Cat# 46890BF; RRID: AB_2799313
Rabbit monoclonal Anti-human & mouse CD11b (EPR1344) antibody	Abcam	Cat# ab216445; RRID: AB_2864378
Rabbit monoclonal Anti-human CD14 (EPR3653) antibody	Abcam	Cat# ab214438; RRID: AB_3676100
Rat monoclonal Anti-human FOXP3 (236A/E7) antibody	Standard BioTools	Cat# 3155016D; RRID: AB_2910136
Rabbit monoclonal Anti-human TIM3 (D5D5R) antibody	CST	Cat# 45208BF; RRID: AB_2716862
Rabbit monoclonal Anti-human LAG3 (D2G4O) antibody	CST	Cat# 15372BF; RRID: AB_2798739
Mouse monoclonal Anti-human CD31 (89C2) antibody	CST	Cat# 3528BF; RRID: AB_2160882
Rabbit monoclonal Anti-human IDO-1(D5J4E) antibody	CST	Cat# 86630BF; RRID: AB_2636818
Rabbit monoclonal Anti-human Ki67 (D2H10) antibody	CST	Cat# 9027BF; RRID: AB_2636984
Rabbit monoclonal Anti-human VISTA (D1L2G) antibody	CST	Cat# 64953BF; RRID: AB_2799671
Rabbit monoclonal Anti-human β 2-microglobulin (D8P1H) antibody	CST	Cat# 12851BF; RRID: AB_2716551
Rabbit monoclonal Anti-human PD-1 (EPR4877) antibody	Standard BioTools	Cat# 3165039D; RRID: AB_3106909
Rabbit monoclonal Anti-human CD8a (D8A8Y) antibody	CST	Cat# 85336BF; RRID: AB_2800052

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Rabbit monoclonal Anti-human CD33 (SP266) antibody	Abcam	Cat# ab238784; RRID: AB_3676111
Rabbit monoclonal Anti-human B7-H3 (D9M2L) antibody	CST	Cat# 14058BF; RRID: AB_2750877
Rabbit monoclonal Anti-human CD45 (EP322Y) antibody	Abcam	Cat# ab214437; RRID: AB_3096032
Rabbit monoclonal Anti-human CD94 (EPR21003) antibody	Abcam	Cat# ab238166; RRID: AB_2920906
Rabbit monoclonal Anti-human CD19 (D4V4B) antibody	CST	Cat# 90176BF; RRID: AB_2800152
Rabbit monoclonal Anti-human CD3e (D7A6E) antibody	CST	Cat# 85061BF; RRID: AB_2721019
Rabbit monoclonal Anti-human CD4 (EPR6855) antibody	Abcam	Cat# ab181724; RRID: AB_2864377
Mouse monoclonal Anti-human Cytokeratin (AE1/AE3) antibody	Abcam	Cat# ab80826; RRID: AB_1640401
Rabbit monoclonal Anti-human & mouse CTLA4 (CAL49) antibody	Abcam	Cat# ab251599; RRID: AB_2905651
Rabbit monoclonal Anti-human & mouse NaKATPase (EP1845Y) antibody	Abcam	Cat# ab167390; RRID: AB_2890241
Mouse monoclonal Anti-dsDNA (35I9 DNA) antibody	Abcam	Cat# ab27156; RRID: AB_470907
Rabbit monoclonal Anti-mouse Vimentin (EPR3776) antibody	Abcam	Cat# ab193555; RRID: AB_2814713
Rabbit monoclonal Anti-mouse CD31 (EPR17259) antibody	Abcam	Cat# ab225883; RRID: AB_2943140
Rabbit monoclonal Anti-mouse CD44 (EPR18668) antibody	Abcam	Cat# ab232556; RRID: AB_3676114
Rabbit monoclonal Anti-mouse CD11c (EPR21826) antibody	Abcam	Cat# ab240558; RRID: AB_3676115
Mouse monoclonal Anti-mouse ECAD (4A2) antibody	Abcam	Cat# ab233766; RRID: AB_3676116
Rabbit monoclonal Anti-mouse β 2-microglobulin (EPR16774) antibody	Abcam	Cat# ab232361; RRID: AB_3676117
Rabbit monoclonal Anti-mouse CD326 (EPR20533-63) antibody	Abcam	Cat# ab228876; RRID: AB_3676118
Rabbit monoclonal Anti-mouse ICOS (EPR20560) antibody	Abcam	Cat# ab225577; RRID: AB_3676119
Rabbit monoclonal Anti-mouse CCR2 (EPR20844-15) antibody	Abcam	Cat# ab273061; RRID: AB_3676120
Rabbit monoclonal Anti-mouse PD-L1(D5V3B) antibody	CST	Cat# 64988BF; RRID: AB_2799672
Rabbit monoclonal Anti-mouse CD49b (EPR5788) antibody	Abcam	Cat# ab271894; RRID: AB_3676122
Rabbit monoclonal Anti-mouse TIM-3 (EPR22241) antibody	Abcam	Cat# ab242080; RRID: AB_3676123
Rabbit monoclonal Anti-mouse SPC (EPR19839) antibody	Abcam	Cat# ab222929; RRID: AB_3676124
Rabbit monoclonal Anti-mouse F4/80 (SP115) antibody	Abcam	Cat# ab240946; RRID: AB_3676125
Rabbit monoclonal Anti-mouse Granzyme-B (EPR22645-206) antibody	Abcam	Cat# ab255868; RRID: AB_3676126
Rabbit monoclonal Anti-mouse CD25 (EPR22588-18) antibody	Abcam	Cat# ab255858; RRID: AB_3676127

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Rabbit monoclonal Anti-mouse CD4 (CAL4) antibody	Abcam	Cat# ab251608; RRID: AB_3676128
Rabbit monoclonal Anti-mouse iNOS (SP126) antibody	Abcam	Cat# ab239990; RRID: AB_2910585
Rabbit monoclonal Anti-mouse NKP46 (EPR23097-35) antibody	Abcam	Cat# ab267792; RRID: AB_3676129
Rabbit monoclonal Anti-mouse CD8a (CAL38) antibody	Abcam	Cat# ab251609; RRID: AB_3676130
Rabbit monoclonal Anti-mouse Ly-6G (EPR22909-135) antibody	Abcam	Cat# ab261916; RRID: AB_3676131
Rabbit monoclonal Anti-mouse Arginase (EPR22033-369) antibody	Abcam	Cat# ab259271; RRID: AB_3676132
Rabbit monoclonal Anti-mouse PAX5 (D7H5X) antibody	lonpath	Cat# 716610; RRID: AB_3676133
Rabbit monoclonal Anti-mouse TTF-1(EPR5955(2)) antibody	Abcam	Cat# ab227574; RRID: AB_3676135
Rabbit monoclonal Anti-mouse Foxp3 (EPR22102-37) antibody	Abcam	Cat# ab244242; RRID: AB_3676136
Rabbit monoclonal Anti-mouse RAGE (EPR21171) antibody	Abcam	Cat# ab228861; RRID: AB_3094831
Rabbit monoclonal Anti-mouse CD3e (SP162) antibody	Abcam	Cat# ab245731; RRID: AB_3676137
Rabbit monoclonal Anti-mouse Ki-67 (SP6) antibody	Abcam	Cat# ab197547; RRID: AB_2924695
Rabbit monoclonal Anti-mouse CD21 (EP3093) antibody	Abcam	Cat# ab271855; RRID: AB_3676138
Rabbit monoclonal Anti-mouse CD206 (E6T5J) antibody	lonpath	Cat# 717404; RRID: AB_3676134
Rabbit monoclonal Anti-mouse CD45 (EPR20033) antibody	Abcam	Cat# ab229292; RRID: AB_3676139
InVivoMAb anti-IgG2a (2A3)	BioXCell	Cat# BE0089; RRID: AB_1107769
InVivoMAb anti-TIM-3 (RMT3-23)	BioXCell	Cat# BE0115; RRID: AB_10949464

Bacterial and virus strains

Ad5CMVCre	Carver College of Medicine	Cat# VVC-U of Iowa-5
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Biological samples

The cohort specimens sourced from New York University Langone Health (NYULH) and Nagasaki Hospital in Japan, all patient information is included in Table S1	N/A	N/A
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Chemicals, peptides, and recombinant proteins

Urethane	Sigma	Cat# U2500-100G
PBS, pH 7.4	Thermo Fisher	Cat# 10010023
Dispase	Stemcell	Cat# 07913
Deoxyribonuclease I	Sigma	Cat# D4513-1VL
Ammonium Chloride Solution	Stemcell	Cat# 07850
Collagenase A	Sigma	Cat# C0130-1G
Hyaluronidase	Sigma	Cat# H3506-1G
Antibody Diluent	CST	Cat# 8112L
Cell-ID™ Intercalator-Ir	Standard BioTools	Cat# 201192A
Maxpar Water	Standard BioTools	Cat# 201069

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Maxpar PBS	Standard BioTools	Cat# 201058
EDTA+G113 Buffer pH9 10X	Agilent Technologies	Cat# S236784-2
Spectral DAPI	PerkinElmer	Cat# FP1490
Ruthenium tetroxide, 0.5% stabilized aqueous solution	Polysciences, Inc.	Cat# 18253-5
ProLong™ Diamond Antifade Mountant	Invitrogen	Cat# P36961
Dewax	Leica	Cat# AR9222
Epitope Retrieval 2 (pH9 EDTA buffer)	Leica	Cat# AR9640
Wash Buffer	Leica	Cat# AR9590
Fetal Bovine Serum	Sigma-Aldrich	Cat# F8318
Nuclease-Free Water (not DEPC-Treated)	Life Technologies	Cat# AM9937
Albumin, Bovine Serum, 10% Aqueous Solution, Nuclease-Free	Sigma	Cat# 126615-25ML
Protector RNase inhibitor 2000 u	Sigma	Cat# 3335399001
D1000 ScreenTape	Agilent Technologies	Cat# 5067-5582
D1000 Sample Buffer	Agilent Technologies	Cat# 5067-5602
RPMI 1640 Medium	Gibco	Cat# C11875500CP

Critical commercial assays

Polymer Refine Detection kit	Leica	Cat# DS9800
Maxpar Praseodymium Chloride 141Pr—50 mM	Standard BioTools	201141A
Maxpar Neodymium Chloride 142Nd, 143 Nd, 144Nd, 145Nd, 146Nd, 148Nd, 150Nd—50 mM	Standard BioTools	201142A, 201143A, 201144A, 201145A, 201146A, 201148A, 201150A
Maxpar Samarium Chloride 147Sm, 149Sm, 152Sm, 154Sm—50 mM	Standard BioTools	201147A, 201149A, 201152A, 201154A
Maxpar Europium Chloride 151Eu, 153Eu—50 mM	Standard BioTools	201151A, 201153A
Maxpar Gadolinium Chloride 155Gd, 156Gd, 158Gd—50 mM	Standard BioTools	201155A, 201156A, 201158A,
Maxpar Terbium Chloride 159Tb—50 mM	Standard BioTools	201159A
Maxpar Gadolinium Chloride 160Gd—50 mM	Standard BioTools	201160A
Maxpar Dysprosium Chloride 161Dy, 162Dy, 163Dy, 164Dy—50 mM	Standard BioTools	201161A, 201162A, 201163A, 201164A
Maxpar Holmium Chloride 165Ho—50 mM	Standard BioTools	201165A
Maxpar Erbium Chloride 166Er, 167Er, 168Er, 169Er, 170Er—50 mM	Standard BioTools	201166A, 201167A, 201168A, 201169A, 201170A
Maxpar Ytterbium Chloride 171Yb, 172Yb, 173Yb, 174Yb, 176Yb—50 mM	Standard BioTools	201171A, 201172A, 201173A, 201174A, 201176A
Maxpar Lutetium Chloride 175Lu—50 mM	Standard BioTools	201175A

Deposited data

Human IMC mcd raw data	This paper	syn61802885
Mouse IMC mcd raw data	This paper	syn61811851
Mouse Single-cell RNA sequencing raw data	This paper	GSE293720
Mouse Visium Spatial Transcriptomics raw data	This paper	syn61842020
Mouse Xenium 5K raw data	This paper	syn63942456
Human reference genome, GRCh38	Genome Reference Consortium	http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/human/
Mouse reference genome, GRCm39	Genome Reference Consortium	http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/mouse/

Experimental models: Cell lines

N/A	N/A	N/A
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Experimental models: Organisms/strains

Mouse: C56BL/6 WT	Lab preserve	
Mouse: 129S4/Sv Kras ^{G12D/+} GEM	Jackson Laboratory	Strain #:008180
Mouse: 129S4/SvJaeJ	Jackson Laboratory	Strain #:009104

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse: C56BL/6 Kras ^{G12D/+} GEM	Lab preserve	
Mouse: C56BL/6 Kras ^{G12D/+} ; P53 ^{R172H/+} GEM	Lab preserve	
Mouse: C56BL/6 Kras ^{G12D/+} ; Stk11 ^{fl/fl} GEM	Lab preserve	
Oligonucleotides		
N/A	N/A	N/A
Recombinant DNA		
N/A	N/A	N/A
Software and algorithms		
ImageScope v12.4.3.5008	Leica Biosystems	https://www.leicabiosystems.com/digital-pathology/manage/aperio-imagescope/
Cell Ranger 3.0.0	10x Genomics	https://github.com/10XGenomics/cellranger
R 4.1.2	R Core Team	https://cran.r-project.org/bin/windows/base/old/4.1.2/
Python 3.8.0	Python Software Foundation	https://www.python.org/downloads/release/python-380/
Seurat 4.3.0	Hao et al. ⁷⁵	https://github.com/satijalab/seurat/releases/tag/v4.3.0
CellChat (v2.1.2)	Jin et al. ⁷⁶	https://github.com/sqjin/CellChat
Monocle 3	Trapnell et al. ³⁰	https://github.com/cole-trapnell-lab/monocle3
DeepCell 0.11.0	Bannon et al. ⁷⁷	https://github.com/vanvalenlab/deepcell-tf/releases/tag/0.11.0
MATLAB R2022b	MathWorks	https://www.mathworks.com/products/new_products/release2022b.html
imcRtools 3.15	Bioconductor	https://bioconductor.org/news/bioc_3_15_release/
SpatialExperiment	Righelli et al. ⁷⁸	https://github.com/drighelli/SpatialExperiment
Cytomapper 1.2.0	Eling et al. ⁷⁹	https://github.com/BodenmillerGroup/cytomapper/tree/v1.2.0
steinbock v0.13.5	Windhager et al. ⁸⁰	https://github.com/BodenmillerGroup/steinbock/releases/tag/v0.13.5
Tidymverse 1.3.2	Wickham et al.	https://tidymverse.tidyverse.org/
CATALYST 1.30.2	Chevrier et al. ⁸¹	https://github.com/HelenaLC/CATALYST
scikit-image 0.18.3	Scikit-image core team	https://scikit-image.org/docs/stable/release_notes/release_0.18.html
scanpy 1.9.1	Wolf et al. ⁸²	https://scanpy.readthedocs.io/en/1.9.x/
opencv-python 4.5.5.64	OpenCV	https://github.com/opencv/opencv-python/releases/tag/64
matplotlib 3.6.3	Matplotlib development team	https://matplotlib.org/3.6.3/
xtiff 0.7.8	Bodenmiller Group	https://github.com/BodenmillerGroup/xtiff/releases/tag/v0.7.8
pycontour 1.4.0	Chen et al.	https://github.com/PingjunChen/pycontour/releases/tag/v1.4.0
Miniconda 3	Continuum Analytics	http://repo.continuum.io/miniconda/Miniconda3-py38_4.8.2-Linux-x86_64.sh
Docker	Docker, Inc	https://www.docker.com/

EXPERIMENTAL MODELS AND STUDY PARTICIPANT DETAILS

Study participants

We collected specimens from a cohort sourced from New York University Langone Health (NYULH), with additional samples secured from the Center for Biospecimen Research and Development (CBRD), and Nagasaki Hospital in Japan. The study received approval from the Institutional Review Boards (IRB) at MD Anderson Cancer Center, Nagasaki University Graduate School of Biomedical Sciences, and New York University (Protocol number: 2021-1160). Independent confirmation of the diagnosis was conducted by two lung cancer pathologists through the review of Hematoxylin and Eosin (H&E) slides for each case. [Table S1](#) provides a summary of the data for the 62 patients in our cohort, with 33 of these patients presenting with multiple lesions.

Mouse models

129S4/Sv-Kras^{G12D/+} (K) (No:008180) and 129S4 wild-type mice (No: 009104) were purchased from The Jackson Laboratory, while C57BL6-Kras^{G12D/+} (K), C57BL6-Kras^{G12D/+}p53^{R172H/+} (KP), and C57BL6-Kras^{G12D/+}Lkb1^{flox/flox} (KLL) mice were lab maintained. All animals were housed in colony cages under pathogen-free conditions at the MD Anderson Research Animal Support Facility. The mice were kept at an ambient temperature of 20 ~ 26°C and a humidity range of 30 ~ 70% with a 12-hour light-dark cycle. All animal experiments were conducted following Institutional Animal Care and Use Committee-approved protocols (Approval number: 00001217-RN03).

METHOD DETAILS

Mouse tissue collection and processing

For carcinogen-induced tumor models (CITMs), we used a Urethane-induced mouse model. Specifically, 129S4 wild-type mice received intraperitoneal injections of Urethane at a dosage of 1 mg/g (body weight) three times over 8 days when they were 6 weeks old. 73 mice were sacrificed at 1, 2, 4, 7, 14, 20, 30, and 40 weeks after Urethane administration, with a 0-week timepoint for mice that received no treatment. We collected both normal lung and lung tumor tissues for downstream analysis. For genetically engineered mouse model (GEMMs), 129S4 K, C57BL/6 K, C57BL/6 KP, and C57BL/6 KLL mice were intranasally instilled with 2.5×10^7 pfu of Ad5-CMV-Cre recombinase adenovirus at the age 6 weeks old. For 129S4 K mouse model, 60 mice were sacrificed at 1, 2, 4, 7, 9, 15, 20, and 30 weeks after adenovirus administration, with a 0-week timepoint for mice that received no adenovirus administration. For C57BL6 K mouse model, 56 mice were sacrificed at 1, 2, 4, 6, 10, 18, and 30 weeks after adenovirus administration, with a 0-week timepoint for mice that received no adenovirus administration. For C57BL6 KP mouse model, 55 mice were sacrificed at 1, 2, 4, 6, 10, 18, and 25 weeks after adenovirus administration, with a 0-week timepoint for mice that received no adenovirus administration. For C57BL6 KLL mouse model, 46 mice were sacrificed at 1, 2, 4, 6, 8, 10, and 12 weeks after adenovirus administration, with a 0-week timepoint for mice that received no adenovirus administration. We collected both normal lung and lung tumor tissues for downstream analysis.

Antibody panel creation and first step validation IMC antibody conjugation

A human antibody panel was designed to specifically target epitopes associated with lung cancer, in addition to markers for cell cycle regulation and immune checkpoints. This panel was also designed to distinguish between epithelial, stromal, and immune cell types. Clone information is available in [key resources table](#). To ensure the reliability of these antibodies, a series of validation steps were carried out. Initially, all antibodies listed in [key resources table](#) underwent testing via immunohistochemistry (IHC). For immune markers, this testing involved a tissue microarray (TMA) constructed using various lymphoid tissues, including spleen, tonsil, and lymph nodes. Lung cancer tissues were used ([Figure S2A](#)) for related markers. Subsequently, a qualified pathologist reviewed the results of these tests to ensure that the expression patterns matched existing literature and that the signal intensity was consistently high. Only antibodies that met these criteria successfully passed our quality control assessment. A mouse antibody panel was designed to specifically target epitopes associated with mouse lung cancer ([key resources table](#)), similar validation and pathological review were performed, and only antibodies that meeting these criteria successfully passed our quality control assessment.

IMC antibody conjugation and second step validation

Antibodies that passed the initial validation were subsequently labeled with metals using the MaxPar X8 Multimetal Labeling Kit (Standard BioTools) following the manufacturer's recommended protocol. After the conjugation process, all antibodies underwent reevaluation by IMC to ensure that their specificity remained intact despite the labeling. The IMC staining results were subjected to validation by another experienced pathologist. To determine the optimal antibody concentrations, we conducted tests using various concentrations for each antibody. Finally, we applied the full antibody panel to stain tissue arrays containing lymphoid tissues and various types of cancer tissues, validating both positive and negative staining patterns.

IMC preparation and staining

Formalin-fixed paraffin-embedded (FFPE) slides were deparaffinized at 56°C overnight in an oven (Thermo Scientific). Tissue section rehydration was carried out in a fume hood with the following steps: three washes with metal-free Xylene, followed by two washes with 100% metal-free Ethanol, two washes with 95% metal-free Ethanol, two washes with 70% metal-free Ethanol, one wash with

metal-free ddH₂O, and one wash with metal-free Phosphate-buffered saline (PBS), each for 10 minutes. The antigen or epitope retrieval is performed with EZ-Retriever v 3.0 microwave. A solution containing 250ml of 1x volume for the holder with Dako target retrieval pH9 (10x; Ref.S2367) was prepared. The microwave was set to reach 99°C for 10 minutes and repeated for two cycles. The slide tank was removed from the microwave and left at room temperature for 15 minutes after antigen or epitope retrieval. Subsequently, slides were washed with metal-free ddH₂O twice and metal-free PBS once. Slides were stained with a cocktail containing metal-tagged antibodies and maintained in a humidified chamber overnight at 4°C. All conjugations were performed by the Department of Translational Molecular Pathology at MD Anderson Cancer Center (MDACC) using the Maxpar Conjugation Kits (Standard BioTools). Information about the antibodies used is available in [Table S1](#). Following staining, slides were washed with metal-free PBS three times, each for 5 minutes. Nuclei were counterstained with a 0.625 μ M isidium solution at room temperature for 30 minutes, followed by three washes with metal-free PBS each for 5 minutes. Tissue structures were counterstained with 0.0005% Ruthenium at room temperature for 4 minutes, and then washed with metal-free PBS three times each for 5 minutes. Dehydration of the slides was achieved with two washes of metal-free ddH₂O, followed by one wash each of metal-free 70% ethanol and metal-free 100% ethanol. The slides were then air-dried under a hood for at least 15 minutes and stored properly at 4°C before and after data acquisition. Tissue ablation was performed using the Standard BioTools Hyperion imaging mass cytometer in the MDACC North Campus Flow Cytometry and Cellular Imaging Core Facility.

ROI selection and IMC imaging acquisition

For the human IMC data, ROIs were selected based on the H&E staining results ([Figure 1B](#)). Data acquisition was carried out using a Helios time-of-flight mass cytometer (CyTOF) in conjunction with a Hyperion Imaging System (Standard BioTools). Prior to laser ablation, optical images of the slides were obtained using the Hyperion software, and the specific areas for ablation were chosen as previously described. Laser ablation was performed with a resolution of approximately 1 μ m and a frequency of 200 Hz. To ensure consistent performance, the machine underwent daily calibration using a tuning slide that included five metal elements (Standard BioTools). In total, we acquired 1655 ROIs from 123 lung cancer tissue sections. Forty-seven ROIs were excluded due to data corruption, 37 due to inadequate image quality, and 10 because the areas lacked islets. All subsequent analyses were conducted using the remaining 1618 ROIs ([Table S1](#)). For the mouse IMC data, ROIs were selected based on the H&E staining ([Figure S6D](#)), data acquisition process was the same with human. In total, we acquired 600 ROIs, 6 ROIs were excluded due to data corruption, 3 due to inadequate image quality, and 2 because the areas lacked islets. All subsequent analyses were conducted using the remaining 589 ROIs ([Table S5](#)).

IMC data preprocessing

The acquired human IMC data was first converted to TIFF format using the MCD viewer (Standard BioTools) ([Figure S1A](#)) for further analysis. To mitigate channel crosstalk occurring in mass cytometry experiments due to minor isotopic impurities, we initially immobilized all metal-conjugated antibodies onto an agarose-coated glass slide. Subsequently, we quantified the isotopic composition through IMC. To address the issue of crosstalk, we harnessed the Bioconductor CATALYST⁸¹ package to generate a ‘spillover matrix’ from this dataset ([Figure S1D](#)). This matrix enabled us to apply a non-negative least-squares regression model via CATALYST⁸¹ for cross-channel spillover correction in single-cell expression data. Furthermore, we performed noise reduction by filtering out sparse and pixelated signals. Additionally, we conducted aggregate filtering to remove antibody aggregates recognized as minuscule, connected components in the image ([Figure S1A](#)). The acquired mouse IMC data was performed with similar procession.

Single-cell segmentation

Cell segmentation was performed on pre-processed images using deep learning-based software Mesmer published by Greenwald et al.⁷³ The input to Mesmer is a two-channel image containing a nuclear marker and a membrane or cytoplasmic marker to accurately delineate single cell boundaries. We adopted Ir191 as the cell nuclear channel and a combination channel of Pan-CK, B2M as the membrane channel, as input for Mesmer. To more effectively capture the range of cell shapes and morphologies present in LUAD, we generated two distinct segmentation parameter sets optimized for non-epithelial and epithelial cells, and further combined the outcome for the final cell segmentation. The non-epithelial setting used a radial expansion of two pixels from the detected nuclear border to generate cell objects, and a stringent threshold for splitting cells. The epithelial setting used a radial expansion of three pixels and a more lenient threshold for splitting cells. We then combined these masks using a post-processing step that gave preference to the epithelial segmentation objects, overriding stromal-parameter-detected objects in the same area.

Cell clustering

Cell clustering was performed in two stages: building an integrated reference dataset using subset of the original data and annotating the rest of the data using this reference. First, we identify two batches of data and integrate them into a shared reference so that cells from the same group will cluster together. Specifically, we used all cell markers to find anchors between the two batches by determining a shared low-dimensional space using canonical correlation analysis (CCA). The identified anchors are used to construct the integrated dataset according to the strategy outlined in.⁸³ For the human IMC data, we next standardized expression values for the cell markers and select only the lineage cell markers including Pan-CK, CD31, aSMA, CD45, CD3E, CD4, CD8A, CD19, CD94, FOXP3, CD11B, CD11C, CD14, MPO, CD68, CD33 to prioritize for cell clustering. The Louvain community detection algorithm was then applied on principal components determined on the selected cell markers resulting in 14 clusters. To assign descriptive

labels to these clusters, we used mean marker expression and determine cell types belonging to two general groups (tumor cells and immune cells). Here we used function markers including Ki67, HLA-DR, B2M, CD45RO, ICOS, GZMB, TIM3, LAG3, VISTA, PD-L1, PD-1, TIGIT, IDO-1, B7-H3, CTLA-4, CD73, CD163. For the mouse IMC data, we standardized expression values for the cell markers and select only the lineage cell markers including CD326, Pan-CK, TTF-1, SPC, RAGE, Vimentin, a-SMA, CD31, CD45, CD3E, CD4, FOXP3, CD8A, PAX5, CD11B, CCR2, ARG, LY6G, CD11C, F480 to prioritize for cell clustering. The Louvain community detection algorithm was then applied on principal components determined on the selected cell markers resulting in 17 clusters. To assign descriptive labels to these clusters, we used the mean marker expression to and determine cell types belonging to two general groups: epithelial cells and immune cells). Here we used function markers including CD44, ECAD, ICOS, PD-L1, CTLA4, TIM-3, GZMB, CD25, INOS, CD127, Ki67, CD21, CD206. After determining the cell types for the references data, we mapped the individual query sets onto the reference and transferred the cell type labels from the reference to the query sets using the method described previously.⁸³

Cell-cell pairwise interaction

Because the distance between the nucleus of neighboring cells varies depending on their size and the degree of cellular agglomeration, we adopted the Delaunay triangulation^{17,84} to identify pairs of cells that are most likely in physical contact. We modeled the cell interaction network based on graph theory,⁸¹ with vertices representing cells and edges representing direct contacts between cells. Because the contact frequencies between different cell types are correlated with these cell type proportions, we used two parallel methods to obtain contact enrichment scores. Value 1 would indicate perfect assortative mixing, where contacts occur only between cells from the same type. Value 0 would indicate random mixing between cell types. A negative value would indicate disassortative mixing, a preference for contacts between cells from different types.

Neighborhood identification

To generate cellular neighborhoods (CN), we used the “window capturing” strategy, which involves counting number of cells (n) in closest proximity to a given cell as described previously.¹⁴ Each window then was represented as a frequency vector consisting of the types of X (as indicated) closest cells to a given cell. After obtaining all the window vector for each cell, the cells were clustered using Scikit-learn (version 1.3.2, a software machine-learning library for Python) Minibatch K-means clustering algorithm (version 0.24.2) with the default batch size of 100 and the random state of 0. Every cell was subsequently assigned to a CN type based on its window vector. The prevalence of each neighborhood in each core was normalized so that the sum of neighborhood prevalence for that core was 1.0. Values were then z-scored and cores with a z-score above or equal to 0 and below 0 were compared for survival outcomes.

Feature extraction

Based on segmented and recognized cells inside each ROI, we extracted four groups of features (Table S2): CT/CN composition (proportion and density), CT/CN morphology, CT/CN state, and CT/CN interaction. The CT/CN proportion of each ROI was measured by dividing the number of one cell subtype by the total number of cells inside, which assessed the cells’ relative abundance. The CT/CN density of each ROI was measured by dividing the number of one cell subtype by the occupied area, which evaluated the compactness of cells. Both CT/CN proportion and density (composition) were independent measurements of one cell subtype without consideration of the spatial arrangement among different cell subtypes which described in our previous study.⁸⁵ Morphological features, including area, eccentricity, major and minor axis lengths, were measured for investigating CT/CN morphologies, measured by the steinbock⁸⁰ Python package (version v0.16.0). The CT/CN state features was evaluated by each CT/CN functional markers’ average expression per cell. CT/CN interaction quantifications were performed as previous elaborated in the cell-cell pairwise interaction, and interaction feature were extracted based on their interaction values at ROIs level.

Quantifying ITH in pathological subtypes using IMC-derived cellular attributes

In the calculation of intratumoral heterogeneity (ITH) for each patient within each pathological subtype, we first obtained 38 attributes across 14 cell types from the IMC images of each Region of Interest (ROI). The ITH for specific features Proportion, Density, State, Morphology, and Interaction was computed as follows: For each feature, the ITH was derived from the average Euclidean distances of the corresponding attributes across pairwise comparisons of ROIs. Specifically, Proportion ITH utilizes the ‘Proportion’ attribute, Density ITH was based on the ‘Density’ attribute, and Morphology ITH involves attributes such as Area, MajorAxisLength, MinorAxisLength, and Eccentricity. For State ITH, we considered the expression levels of 17 proteins, including Ki67, HLADR, B2M, CD45RO, ICOS, GZMB, TIM3, LAG3, VISTA, PDL1, PD1, TIGIT, IDO1, B7H3, CTLA4, CD73, and CD163. Interaction ITH was calculated using the interactions among 14 different cell types.

Trajectory analysis

For trajectory analysis, we used Monocle 3.^{30,86} First, samples were processed using the standard Seurat approach⁷⁵ which include scaling, normalization, variable feature selection and PCA analysis. We used Monocle’s default parameter settings to ensure that our trajectory analysis results were reproducible. An embedding of the samples into a UMAP subspace was then determined from which the trajectory graph was inferred. To model the evolution of tumor from one stage to another, we used domain knowledge and set the starting of the trajectory to be the cluster containing the normal samples. Finally, to further determine the features that govern the

evolution of tumor from one stage to another along the trajectory, we tested features for differential expression and found modules of co-expressed genes across the trajectory.

Feature importance analysis

To identify differentially expressed (DE) features that facilitates tumor evolution from Normal to AAH, AAH to AIS, AIS to MIA and MIA to IAC, we performed DE analysis by comparing this pairs of groups using Wilcoxon rank sum test.⁸⁷ We set a cutoff such that DE analysis was performed only on features that associates with at least 25% of samples in both the compared stages. Feature importance were then computed using the returned adjusted p-values of all DE features as to identify differentially expressed (DE) features that facilitates tumor evolution from Normal to AAH, AAH to AIS, AIS to MIA and MIA to IAC, we perform DE analysis by comparing this pairs of groups using Wilcoxon rank sum test.⁸⁷ We set a cutoff such that DE analysis was performed only on features that associates with at least 25% of samples in both the compared stages. Feature importance was then computed using the returned adjusted p-values of all DE features as

$$f_i = - \frac{\log(\bar{p}_i)}{\sum_{k=1}^n \log(\bar{p}_k)}$$

where $\bar{p}_i$ denotes the adjusted p-value for feature i returned from the DE test.

Tissue preparation and spatial transcriptomics

Normal and tumor tissues from mouse lungs were fixed in 10% formalin at room temperature for 24 hours (using a fixative volume 5-10 times that of tissue volume). Fixed tissues were transferred to 70% ethanol for temporary storage at 4°C. Paraffin embedding was conducted by the MD Anderson cancer center (MDACC) Research Histology Core Laboratory. Formalin-Fixed Paraffin-Embedded (FFPE) Blocks were cut into 5 μm thick sections using a pre-cooled, RNase-free microtome. These sections were then transferred onto Visium Spatial Gene Expression slides (10x Genomics), which were pre-treated by floating them on a water bath at 43°C. Following sectioning, the slides were dried at 42°C in a Thermal Cycler (SimpliAmp™ Thermal Cycler, Thermo Fisher Scientific) for 3 hours, following the manufacturer's instructions. The slides were placed in a slide mailer, sealed with parafilm, and stored overnight in a refrigerator at 4°C. The slides were then deparaffinized, fixed, stained with hematoxylin and eosin (H&E), and imaged at 5X magnification using a Leica DM5500 B microscope (Leica Microsystems). Tile scans of the entire array were acquired using the Leica Application Suite X (LAS X) and merged. Spatial gene expression libraries were processed according to the manufacturer's instructions (10x Genomics, Visium Spatial Transcriptomic) and sequenced using a NovaSeq 6000 sequencer (Illumina). All H&E staining, imaging, library preparation, and sequencing processes were carried out by the Genomic & RNA Profiling Core at Baylor College of Medicine.

Mouse single cell suspension preparation and sequence

Fresh lung and tumor samples were collected from mice, washed with PBS, and minced into ~1 mm³ pieces in RPMI-1640 medium (Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS, Gibco). The tissues were enzymatically digested with a solution of 1 mg/ml collagenase A, 0.4 mg/ml hyaluronidase, and BSA fraction V (1:5) for 1 ~ 2 hours at 37°C on a rotor, following the manufacturer's instructions. The digested samples were centrifuged at 350g for 5 minutes, the supernatant was removed, and 1 ~ 5 ml of pre-warmed Trypsin-EDTA was added. The cells were resuspended and gently pipetted for 1-3 minutes, resulting in a stringy consistency due to the lysis of dead cells and released DNA. After adding 10 ml of cold RPMI-1640 without phenol red supplemented with 2% FBS, the samples were centrifuged again at 350g for 5 minutes, and the supernatant was discarded. If cell clumps persisted, 5 ml of pre-warmed Dispase (5 U/ml) and 50 μL of DNase I solution (10 mg/ml in 0.15 M NaCl) were added, followed by 1 minute of pipetting. For persistent stringiness, an additional 50 μL of DNase I was used. The dissociated cells were diluted with 10 ml of cold RPMI-1640 without phenol red (2% FBS), filtered through a 40 μm cell strainer, and centrifuged at 450g for 5 minutes. To remove red blood cell contamination, the cell pellet was treated with a 1:4 mixture of cold RPMI-1640 (2% FBS) and ammonium chloride solution, centrifuged, and resuspended in PBS with 1% BSA. The final cell suspension, with viability ≥70% and a density of 7×10^5 to 1.2×10^6 cells/ml, was loaded onto the Chromium Single Cell Controller (10x Genomics) for single-cell gel bead-in-emulsion generation. scRNA-seq libraries were prepared using the Single Cell 3' Library and Gel Bead Kit v3.1 and sequenced on the NovaSeq 6000 platform (Illumina).

Single-cell RNAseq analysis

Raw scRNA-seq data were processed with Cell Ranger, followed by rigorous quality control. Cells with fewer than 200 or more than 5,000 unique features, or with mitochondrial content exceeding 15%, were excluded. Batch effects were addressed using Seurat's integration pipeline to ensure data consistency. Clusters expressing conflicting lineage markers were identified and removed based on canonical marker gene expression. *Havcr2*⁺ cells were defined as those with normalized expression levels greater than 0.1.

Cell-cell communication

The communications between two cell groups were inferred based on the expression of known ligand-receptor pairs across different cell types, as defined in CellChat⁷⁶ (v2.1.2). Additional ligand-receptor pairs (*Ceacam1_Havcr2*, *Hmgb1_Havcr2*, *Ptdss1_Havcr2* and

Ptdss2_Havcr2) were manually added to CellChat.mouse database. Interactions involving cell groups with fewer than 50 cells were excluded. Communication probabilities and p-values for ligand–receptor interactions between different cell groups were calculated for each time point.

TIM-3 blockade *in vivo*

For 129S4 Urethane model LUAD early stage TIM-3 blockade experiment, 40 mice received intraperitoneal injections of Urethane at a dosage of 1 mg/g (body weight) three times over 8 days when they were 6 weeks old, mice were randomly arranged into two groups (20 IgG treatment, 20 anti-TIM-3 treatment) after urethane administration, the anti-TIM-3 antibody (CD366, Bio X Cell) or IgG2a isotype control (2A3, Bio X Cell) at a dose of 200 μ g per mouse was administered three times/week through intraperitoneal injection (Figure S10A), starting at 4 weeks when hyperplasia was first be observed after Urethane induction (Figure S6B). 4 mice from IgG treatment group, and 2 mice from anti-TIM-3 treatment group died during the treatment. 16 mice from IgG treatment group, and 18 mice from anti-TIM-3 treatment group were sacrificed after 10 weeks treatment, lung tissues were collected to evaluate the TIM-3 blockade efficacy. For 129S4 Urethane model LUAD late stage TIM-3 blockade experiment, 32 mice randomly arranged into two groups (16 IgG treatment, 16 anti-TIM-3 treatment) after urethane administration, the anti-TIM-3 antibody (CD366, Bio X Cell) or IgG2a isotype control (2A3, Bio X Cell) at a dose of 200 μ g per mouse was administered three times/week through intraperitoneal injection (Figure S10C), starting at 30 weeks when adenocarcinoma was first be observed after Urethane induction (Figure S6B). Seven mice from IgG treatment group, and 2 mice from anti-TIM-3 treatment group died during the treatment. 9 mice from IgG treatment group, and 14 mice from anti-TIM-3 treatment group were sacrificed after 10 weeks treatment, lung tissues were collected to evaluate the TIM-3 blockade efficacy.

Mouse xenium 5K assay

Tissue preparation and sectioning were the same with spatial transcriptomics assay. The Xenium spatial transcriptomics data were processed to ensure high-quality input for downstream analysis. The data were normalized and variance-stabilized, followed by scaling and dimensionality reduction with PCA. Clustering was then performed using the Louvain algorithm. Marker expression was further examined to validate cluster identities. Integration with single-cell RNA-seq data was performed by aligning the Xenium spatial single cell dataset with an annotated reference single-cell dataset. Using FindTransferAnchors function from Seurat, anchors were established between the Xenium and single-cell dataset, enabling the transfer of cell type labels from the reference to the Xenium dataset. This integration facilitated the annotation of the spatial transcriptomics clusters with single-cell RNA-seq-informed cell type identities, providing robust phenotypic characterization within the spatial context. To evaluate antigen presentation activity in dendritic cells, we first identified a curated list of genes involved in antigen presentation and immune response pathways. These markers include: *Ciita*, *Tap1*, *Erap1*, *Psmb9*, *Cd80*, *Cd86*, *Cd40*, *Icosl*, *Cd209a*, *Tlr2*, *Tlr3*, *Tlr4*, *Tlr7*, *Tlr9*, *Il12a*, *Il12b*, *Il15*, *Il18*, *Cxcl9*, *Cxcl10*, *Ccl19*, *Irf8*, *Irf4*, *Batf3*, *Stat1*, *Stat3* and *Relb*. The antigen presentation signature score for each dendritic cell was calculated using Seurat's "AddModuleScore" function, which computed the average expression of the selected markers normalized against a control background of randomly selected genes with similar expression profiles. Similarly, a T cell activation signature was constructed to assess the activation state of T cells in the datasets. The curated list of activation-related genes included: *Cd69*, *Cd25*, *Cd40lg*, *Cd28*, *Icos*, *Il2*, *Ifng*, *Tnf*, *Il4*, *Il10*, *Il21*, *Prf1*, *Gzmb*, *Zap70*, *Lat*, *Lck*, *Fos*, *Jun*, *Ctla4*, *Pdcd1*, *Lag3*, *Ccr7*, *Cxcr3*, *Ccl5*; Transcription Factors: *Foxp3*, *Tbx21*, *Gata3*, *Rorc*, *Nfatc1*, *Itgal*, *Itga4*, *Cd3e*, *Cd8a*, *Cd8b*, *Cd4*. Using the "AddModuleScore" function, a T cell activation score was computed for each T cell-based on the average expression of the selected markers, normalized against a similar control background. These computed signature scores provide quantitative measures of antigen presentation and T cell activation, facilitating the investigation of immune activity and functional states across the spatial landscape of the tissues.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism (v.10) (for experimental data), and R (v.4.2.2), RStudio (v.2023.09.1) and Python (v.3.10.2) (for IMC data, ST data and matched clinical variables). Student's t-tests and Wilcoxon rank-sum tests were used for continuous variables. Paired t-tests were used for paired comparisons. Differences with $P < 0.05$ were considered statistically significant.

ADDITIONAL RESOURCES

The major software and algorithms we used in this study but not mentioned in the specific method section included DeepCell (0.11.0),⁷⁷ MATLAB (R2022b), imcRtools (3.15), SpatialExperiment,⁷⁸ Cytomapper (1.2.0),⁷⁹ Tidyverse (1.3.2), scikit-image (0.18.3), scanpy (1.9.1),⁸² opencv-python (4.5.5.64), matplotlib (3.6.3), xtf (0.7.8), pycontour (1.4.0), Miniconda 3, and Docker.