

Mechanisms of Progression and Heterogeneity in Multiple Nodules of Lung Adenocarcinoma

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Lung cancer remains the leading cause of cancer-related death worldwide. Lung adenocarcinoma (LUAD) is thought to be caused by precursor lesions of atypical adenoma-like hyperplasia and may have extensive in situ growth before infiltration. To explore the relevant factors in heterogeneity and evolution of lung adenocarcinoma subtypes, the authors perform single-cell RNA sequencing (scRNA-seq) on tumor and normal tissue from five multiple nodules' LUAD patients and conduct a thorough gene expression profiling of cancer cells and cells in their microenvironment at single-cell level. This study gives a deep understanding of heterogeneity and evolution in early glandular neoplasia of the lung. This dataset leads to **discovery of the changes in the immune microenvironment during the development of LUAD, and the development process from adenocarcinoma in situ (AIS) to invasive adenocarcinoma (IAC)**. This work sheds light on the direction of early tumor development and whether they are homologous.

1. Introduction

Lung cancer remains the leading cause of cancer-related death worldwide. With the use of low-dose computed tomography (LDCT) screening, more and more pulmonary nodules have been diagnosed.^[1] Most patients with pulmonary nodules are diagnosed as lung adenocarcinoma (LUAD), which progresses from adenocarcinoma in situ (AIS) to minimally invasive carcinoma (MIA) then to invasive adenocarcinoma (IAC). Some

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patients are detected as multiple nodules, and different nodules are at different pathological stages.

Lung cancer, like many other cancers, is a heterogeneous disease within cells that is caused by a variety of factors. Single-cell sequencing provides a means to characterize the heterogeneity of cells and opportunities to determine key molecular characteristics that affect clinical outcomes. Genomic intratumoural heterogeneity (ITH) has been identified as a key hallmark of malignancy progression^[2] including lung cancer.^[3] A previous study described that genomic ITH was closely associated with patients' prognosis in LUAD,^[3] whereas substantial heterogeneity and complex interactions of immune cells in tumor microenvironment (TME) were also revealed in early-stage LUAD

with *EGFR* alterations.^[4] It was also previously reported that genomic ITH and immunoediting in TME might have occurred before tumor invasion.^[5] Besides, many researchers analyzed the stromal cells, innate immune cells, tumor microenvironment, T cells, dendritic cells, and fibroblast in lung cancers, and dissected the NSCLC of intra-tumoral myeloid cells plasticity by single-cell RNA-seq (scRNA-seq).^[6,7,8,9,10] Despite that, it still remains unclear whether immune-ITH in TME relates to tumor development in LUAD.

To explore cellular composition and molecular characteristics of LUAD subtypes in patients with multiple nodules, we performed scRNA-seq on tumor and paracancerous tissues from five different LUAD patients. A thorough gene expression profiling of cancer and paracancerous cells at single-cell level was conducted. We also analyzed the cellular network of LUAD at different pathological states to identify cellular interactions and analyzed the heterogeneity and evolution in different nodules. By analyzing the dataset, we would like to show changes in stromal and cancer cells during the disease progression from AIS to MIA and at last to IAC. Studying this process will improve our awareness about LUAD and may help find ways to prevent the progression of LUAD.

2. Results

We analyzed cell types in LUAD by scRNA-seq. Preliminary assumptions about tumor heterogeneity and evolution were

made. Then, we compared the differences between microenvironment and expression patterns of cancer cells in different patients or different nodules from the same patient to determine the exact heterogeneity and evolution of lung adenocarcinoma. The trajectory analysis was also performed to prove the tumor evolution further. The whole process was shown in Figure 1.

2.1. scRNA-seq Revealed Different Cell Types in Lung Adenocarcinoma (LUAD) Nodules

To reveal the differences among cell types in patients with LUAD, we performed the scRNA-seq for multiple cancer nodules and adjacent tissues (Table 1) from five patients (25 different samples). Only 1 (P6) patient was a former smoker.

After data quality control, we obtained a total of 53 610 cells, 24 956 cells (46%) from lung tumor tissues, and 28 654 (54%) cells from adjacent tissues. Twelve clusters were assigned to known cell types through their characteristic marker genes by t-distributed stochastic neighbor embedding (t-SNE) (Figure 2A). These were macrophages (LYZ, MRC1), mast cells (TPSB2, TPSAB1), T cells (CD3D, CD2), plasma cells (CD79A, JCHAIN, MZB1), B cells (MS4A1, CD79A), type 2 alveolar epithelial cells (SFTPB, SFTPC, ABCA3), club cells (SCGB3A2, SCGB1A1), endothelial cells (PECAM1, VWF), epithelial cells (SCGB3A2, TPPP3, PIFO), dendritic cells (CD1C, FCER1A, CLEC9A), cancer cells (NAPSA, NKX2-1), and type 1 alveolar epithelial cells (AGER, CAV1) (Table 2).

The lung is the primary site of immune regulation and can mount a strong and highly regulated immune response to protect the host from pathogen infections. Epithelial cells, dendritic cells, macrophages, neutrophils, eosinophils, B lymphocytes, T lymphocytes, and many other cells can contribute to lung immunity.^[11] An increasing number of studies suggest that stromal cells, such as macrophages, T cells, and fibroblasts, are highly heterogeneous in tumor and healthy lung tissues.^[12,13,14]

We compared the proportions of different types of cells in each patient. Most plasma cells, endothelial cells, dendritic cells, and B cells came from tumor tissues, which implied their potential connections with tumors. Neoplasm might stimulate the infiltration of these cells. While most of the T cells, mast cells, macrophages, and type 1 alveolar epithelial cells came from adjacent normal tissues (Figure 2B). There were apparent differences between tumor and adjacent tissues, especially in macrophages and dendritic cells ($p < 0.05$). The infiltration of T and B cells in all tissues of all patients was low except for the smallest nodule of P6, while that of myeloid cells was high (Figure 2C). In other words, the myeloid cells mainly made up the tumor microenvironment in terms of quantity. We analyzed the distribution of different cellular subpopulations depending on different pathological subtypes: adenocarcinoma in situ (AIS), minimally invasive carcinoma (MIA), and invasive carcinoma (IAC) (Figure 2D). We found that the proportion of T cells was significantly different in MIA, AIS, and IAC ($p < 0.05$). With the development of LUAD from AIS to MIA to IAC, T cells' proportion was gradually decreased. This was consistent with previous reports that stage I LUAD lesions already have significantly altered T cell and NK cell compartments.^[12]

In contrast, the percentage of macrophages and dendritic cells in IAC and MIA was higher than that in AIS (Figure 2D). From Table 1 and Figure 2C, we can also see that the adjacent tissue in patient number 2 (P2) (H13, H14), P3 (H20, H21), P4 (H25, H26, and H27, H28), and P6 (H42, H43) had significantly higher T-cell infiltration than that in the tumor. The patient 6 (P6) had relatively lower T cell infiltration with the pathologic subtypes from AIS to IAC. Other patients showed a similar tendency. According to our data, we found that percentage of tumor-infiltrating T cells was higher in the less invasive pathological subtype, while the percentage of macrophages in tumor tissue was higher in a more invasive pathological subtype. All these data showed that T cells played a role in inhibiting tumor invasion, and other cells like macrophages, regulatory T cells, and cancer-associated fibroblasts act the opposite function.

By analyzing the cell type composition ratios in all five patients, we found heterogeneity among different nodules and between nodules and adjacent normal tissue (Figure 2E). We also used a "Heterogeneity Score" (see Experimental Section) to quantify the intra-tissue heterogeneity (Figure 2F). The highest heterogeneity among different nodules was identified in patient P6 (H38) (Figure 2F), followed by P4 (H27) and P6 (H40), which were all IAC. On the contrary, P2 and P10 had low heterogeneity in general. The heterogeneity was high between the nodules and their corresponding adjacent tissue in each patient.

To identify cellular and molecular shifts in the cascade from normal tissue to cancer, we tried to systematically construct a single-cell transcriptomic network by characterizing the associations between each pair of cancer cells and immune cells (Figure 2G). The color in the network denotes the Pearson correlation coefficient between the centroids of the pair of cell types. As shown in Figure 2G, the most significant correlation coefficient was identified between tumor cells and myeloid cells, tumor cells and endothelial cells, and myeloid cells and alveolar cells (Figure 2G).

Comparing nodules and adjacent tissues, we also found that genes topoisomerase II alpha (*TOP2A*), matrix metalloproteinase 15 (*MMP15*), and MX dynamin-like GTPase 2 (*MX2*), were expressed at a high level in tumor tissues (Figure 2H), which was consistent with previous studies.^[15] *TOP2A* gene encodes a protein of topoisomerase II α , an essential nuclear enzyme for chromosome segregation during mitosis.^[16] MMPs (matrix metalloproteinases) are a family of zinc- and calcium-dependent enzymes involved in the degradation of numerous extracellular matrix components. *MX2* gene belongs to the family of large GTPases.^[15,17] The expression of *TOP2A*, *MMP15*, and *MX2* might be correlated with the genesis of adenocarcinoma.

We found five targeting innate immunity genes by analyzing differentially expressed genes (DEG). They were *APOE*, *C5AR1*, *STAT3*, *CD27*, and *CD47*. The violin plot also showed the expression of these genes in different cells (Figure 2I). *APOE* was mainly expressed in macrophages. As mentioned before, the percentage of macrophages was higher in IAC, so *APOE* might influence the number of macrophages and then promote the tumor invasion. *C5AR1* and *CD47* were similar to *APOE*, while *STAT3* and *CD27* were the opposite. These results were in accordance with previous studies, where *CD27* expressed in

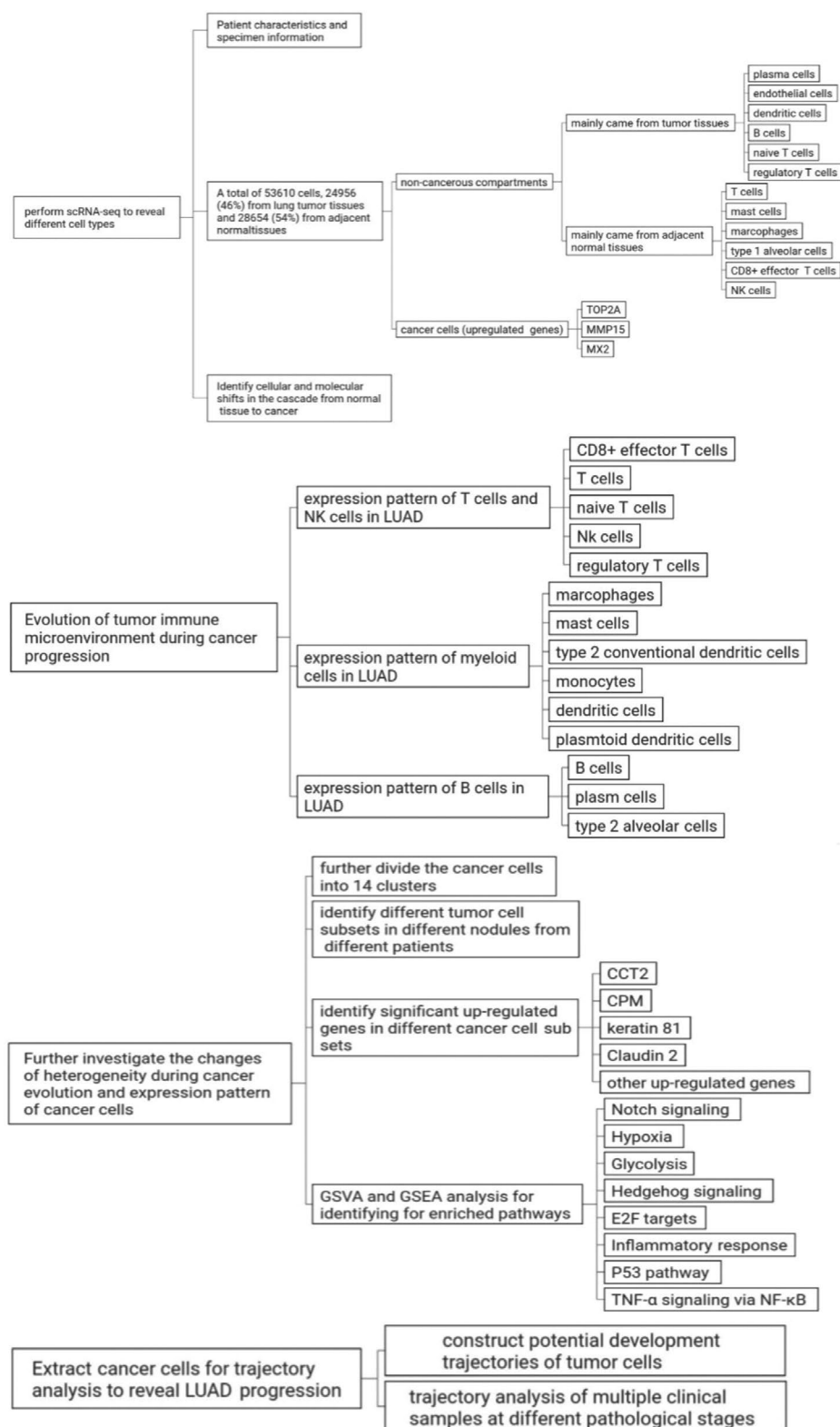


Figure 1. Flow chart describing the whole progress and train of thought.

plasma cells could induce and amplify the immune response. STAT3 is an important transcription factor in immune function. In phagocytes, STAT3 mainly show an anti-inflammatory

effect. Moreover, genes regulated by STAT3 in tumor-associated macrophages encode molecules that contribute to immune suppression.^[18,19] In this part, we showed the differences between

Table 1. Patient characteristics and specimen information.

NO	ID	Sample ID	Specimen species	Gender	Age	Smoke	Pathology	Size[cm]
2	ZX	H11	tumor tissues	female	43	0	MIA	1.2 * 0.7 * 0.7
		H12	adjacent tissues				normal	
		H13	tumor tissues				AIS	0.8 * 0.5
		H14	adjacent tissues				normal	
		H17	tumor tissues				AIS	0.2
3	TSP	H20	tumor tissues	female	53	0	IAC	3.3 * 2.0 * 1.5
		H21	adjacent tissues				normal	
		H22	tumor tissues				MIA	0.7
		H23	adjacent tissues				normal	
4	SLF	H25	tumor tissues	female	70	0	IAC	2.0 * 1.3
		H26	adjacent tissues				normal	
		H27	tumor tissues				IAC	1.3
		H28	adjacent tissues				normal	
6	CYJ	H38	tumor tissues	male	63	1	IAC	1.5 * 1.0 * 1.0
		H39	adjacent tissues				normal	
		H40	tumor tissues				IAC	1.5 * 1.2 * 1.0
		H41	adjacent tissues				normal	
		H42	tumor tissues				AIS	1.1
10	SJP	H43	adjacent tissues	male	69	0	normal	
		H63	tumor tissues				MIA	3.1 * 2.5 * 2.0
		H64	adjacent tissues				normal	
		H65	tumor tissues				MIA	3.0 * 1.5 * 1.0
		H66	adjacent tissues				normal	
		H67	tumor tissues				MIA	2.3 * 1.5 * 1.0
		H68	adjacent tissues				normal	

adenocarcinomas in situ (AIS), microinvasive adenocarcinoma (MIA), invasive adenocarcinoma (IAC)

stromal cells among different patients and different pathological stages in LUAD.

2.2. Explore the Relationship between the Heterogeneity and Evolution of LUAD and the Expression Pattern of Cancer Cells in LUAD

In the tumor microenvironment, since immune cells are related to pathological subtypes of cancer and have an essential impact on cancer prognosis and responses to the treatment, we compared the level of T cells, myeloid cells, and B cells in different pathological subtypes of LUAD.

2.2.1. Expression Pattern of T Cells in LUAD

It has been shown that stage I LUAD lesions already have significantly altered T cell and NK cell compartments.^[12] We extracted all T cells from cancer and adjacent normal tissues and detected a total of 7195 cells (3084 from cancer tissues and 4111 from normal tissues). These cells were divided into five cell subpopulations: CD8⁺ effector T cells (CD3D, NKG7, CD8A), T

cells (CD3D), naive T cells (CD3D, CCR7), NK cells (CD3D⁺, NKG7, FCGR3A,) and regulatory T cells (CD3D, TNFRSF4, CTLA4) (Figure 3A). Among those, CD8⁺ effector T cells, T cells, and NK cells were enriched in normal tissues, and naive T cells and regulatory T cells are enriched in tumors, suggesting a suppressive effect of the tumor on T cells in its microenvironment (Figure 3B). The cancer tissues P6 H38 and P3 H20 (both IAC) were composed of a high percentage of regulatory T cells. The highest ratio of CD8⁺ effector T cells was detected in all P2 tissues and adjacent tissues of P4 H26 and H28. We found that CD8⁺ effector T cells are enriched in adjacent normal tissues of IAC compared with that of AIS and MIA, which suggested their potential role in tumor suppression. Finding methods to attract these cells into tumor tissue might be an alternative strategy for cancer treatment.

We analyzed the proportions of T and NK cell subsets in different nodules of the same patient (Figure 3C). T cell subsets' heterogeneity in nodules of patient P3 and P6 was higher than P2, P4, and P10. Therefore, the heterogeneity between MIA and AIS was lower than MIA and IAC, while between AIS and IAC, it was the highest. Both nodules of P4 were IAC, and the highest proportion of cells was naïve T cells. The ratio of regulatory T cells in P3 H20 (IAC) was above 50%, while of the naïve

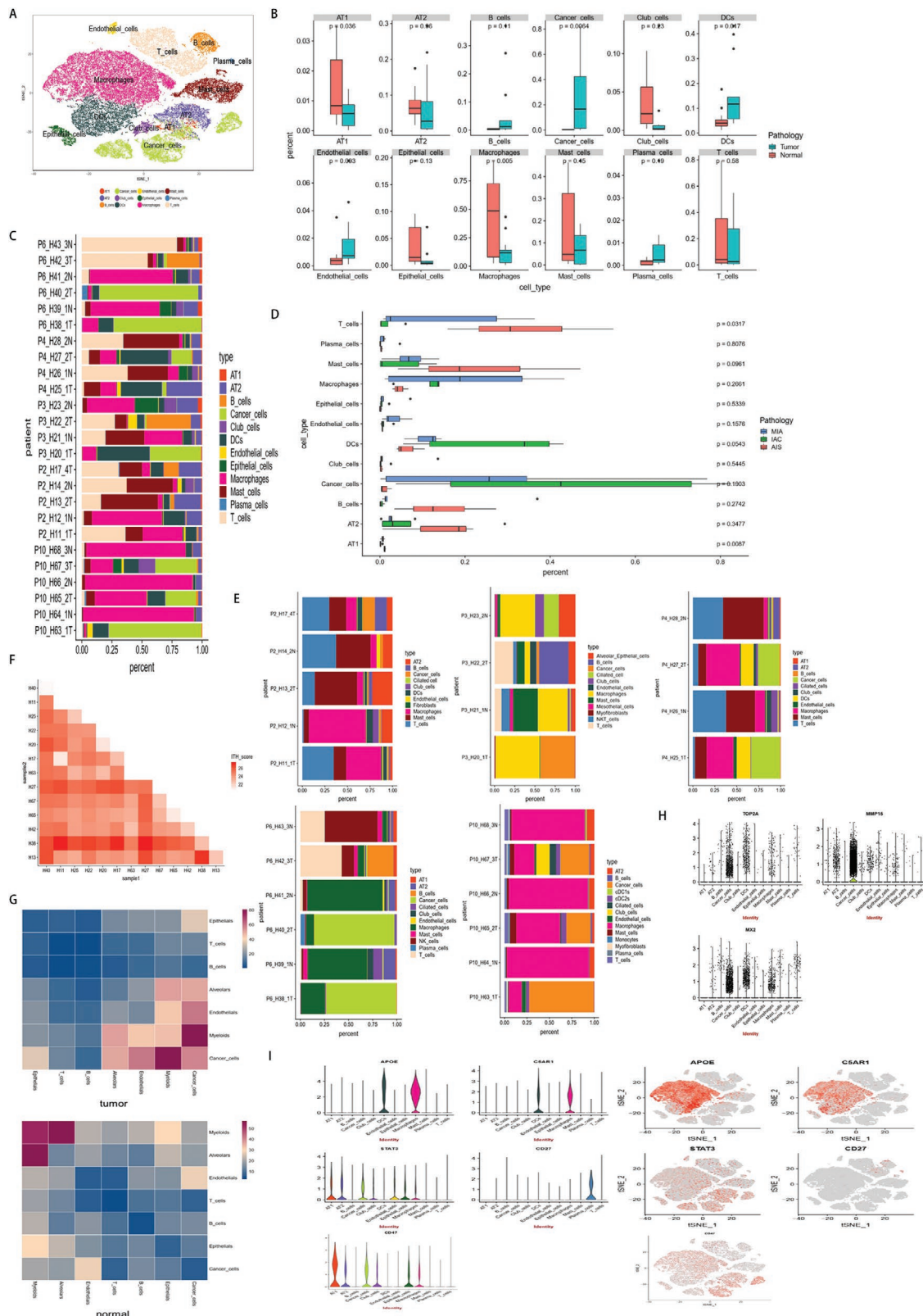


Figure 2. scRNA-seq reveals different cell types in LUAD. A) The t-SNE plot of cells to visualize cell-type clusters. t-SNE was used to divide the cell into thirteen clusters due to their characteristic marker genes. B) The fraction of cells originating from cancer cells and adjacent tissue cells in clusters. The blue one represented the origin of the tumor cells, and the red one demonstrated the origin of the normal cells. C) The fraction of cells originating from each of the five patients in cell types. Different colors demonstrated different cell types, and the numbers in the left are the same as those in

Table 2. Cell types and relevant markers in our study.

Cell subtype	Markers
macrophages	LYZ, MRC1
mast cells	TPSB2, TPSAB1
T cells	CD3D, CD2
plasma cells	CD79A, JCHAIN, MZB1
B cells	MS4A1, CD79A
type 2 alveolar epithelial cells	SFTPB, SFTPC, ABCA3
club cells	SCGB3A2, SCGB1A1
endothelial cells	PECAM1, VWF
epithelial cells	SCGB3A2, TPPP3, PIFO
dendritic cells	CD1C, FCER1A, CLEC9A
cancer cells	NAPSA, NKX2-1
type 1 alveolar epithelial cells	AGER, CAV1

T cells in H22 (MIA) was about 75%. P6 was the only patient with a history of smoking; the primary subtype of H38 was regulatory T cells, and of H40 (both IAC) was regulatory T cells and NK cells, while the content of naïve T cells was shallow in other IAC samples. The main subtypes of P10 H65 and H67 (both MIA) were naïve T cells. In H63 (MIA), the regulatory T cells were more than 50%, and there were no CD8⁺ effector T cells and naïve T cells. We compared the composition of T cell subpopulations in MIA, AIS, and IAC of all patients and found no significant difference. Nodules with the same pathologic subtypes showed low heterogeneity of T cells, while those with different pathologies were high (Figure 3D).

Analysis of hallmark pathway gene signatures highlighted that differential genes of regulatory T cell subset were mainly concentrated in the following pathways: G2M checkpoint pathway and E2F targets pathway (Figure 3E).

CD8⁺ effector T cells and NK cells mainly came from normal tissues, and they were more in MIA than AIS, which might be caused by elevated neoantigen burden that made tumor higher antigenicity to attract more immune cells during the development from AIS to MIA but exact reason still needs to be investigated. Regulatory T cells and naïve T cells mostly came from MIA and IAC tumor tissues.

To sum up, differential genes of regulatory T cells were enriched in the G2M checkpoint pathway, E2F targets pathway, and so on. The T and NK cells' pattern could influence the tumor evolution and progression. The flow chart is in Figure 3F.

2.2.2. Expression Pattern of Myeloid Cells in LUAD

Myeloid cells are a diverse population of immune cells that can sense and respond to tissue damage by clearing injured cells

and facilitating the recruitment of immune effector cells.^[20] A total of 29 376 myeloid cells (normal adjacent tissues 20 118; and cancer tissues 9258) were detected, and eight cell subsets were obtained by clustering. The main subpopulations were macrophages, mast cells, type 2 conventional dendritic cells (cDC2), and monocytes (Figure 4A). Macrophages and DCs play an important role in coordinating immune response and responding to potential threats. In addition to innate immune function, they also can present tumor-associated antigens to T cells.^[21,22]

The proportion of cDC2 was significantly different in normal and cancer tissues (Figure 4B): cDC2 had a significantly higher proportion in tumors than in normal tissues. DCs are competent helper cells that play an essential role in initiating the primary immune response. DCs are potent antigen-presenting cells and have been shown to have a significant impact on clinical outcomes of various cancers.^[23] These results indicated it was possible that tumor antigens could be presented to effector cells. However, the real functional state of cDC2 in the tumor microenvironment and their roles in tumor need further studies to confirm.

In the respiratory system, mast cell activity is involved in maintaining a healthy lung by contributing to innate and adaptive immunity to infection by pathogens.^[24] Mast cells accumulate in an angiogenesis-dependent manner in LUAD. It has been shown that tumor-released vascular endothelial growth factors may be related to mast cell accumulation, and stromal mast cells correlate with angiogenesis and poor outcome in stage I LUAD.^[25] We analyzed the proportions of different myeloid cell subsets in different adjacent tissues and nodules of the same patient (Figure 4C,D). The mast cells of P4 patient were exhausted in tumor tissues, the infiltration of macrophage in advanced LUAD (MIA, AIC) was high, and tumor tissues were enriched with cDC2s (Figure 4C). Macrophage infiltration was high in almost MIA and IAC nodules and adjacent tissues in P6 and P10 patients. Tumor samples H20 and H38 (both AIC) have high macrophage infiltration, and almost no mast cells. Different nodules coming from P4 (both IAC) and P10 (all MIA) have lower heterogeneity than that from P2, P3, and P6. Myeloid cell subtypes correlated with different pathology subtypes of the nodules (Figure 4D). For nodules from the same patient, the ones at earlier pathological stage (AIS) had a higher proportion of mast cells, while the ones at advanced pathologic stage (IAC) had higher proportion macrophage infiltration (P2, P3, and P6) (Figure 4E). In summary, we found that the myeloid subgroup of AIS was dominated by mast cells, while macrophages dominated the MIA and IAC.

Analysis of the hallmark pathway highlighted that the tumor necrosis alpha (TNF- α) signaling via nuclear factor kappa B (NF- κ B), inflammatory response, IL6-JAK-STAT3 signaling, interferon-alpha response, and interferon-gamma response were increased in monocytes (Figure 4F). Interferon-alpha

Table 1. D) Expression pattern of cell type in MIA, IAC, and AIS. In this figure, the percent of different cells in different degrees, is as shown. E) The fraction of cells originating from each of the five patients. F) The Heterogeneity Score of different nodules. G) The single-cell transcriptome network underlying tumor and adjacent tissues of all patients. H) Violin plots showing the expression of marker genes in different cell subsets as indicated in tissues. I) t-SNE plot color-coded for expression (gray to purple) of marker genes for the cell subsets as indicated. Moreover, Violin plots showed the expression distribution of selected genes.

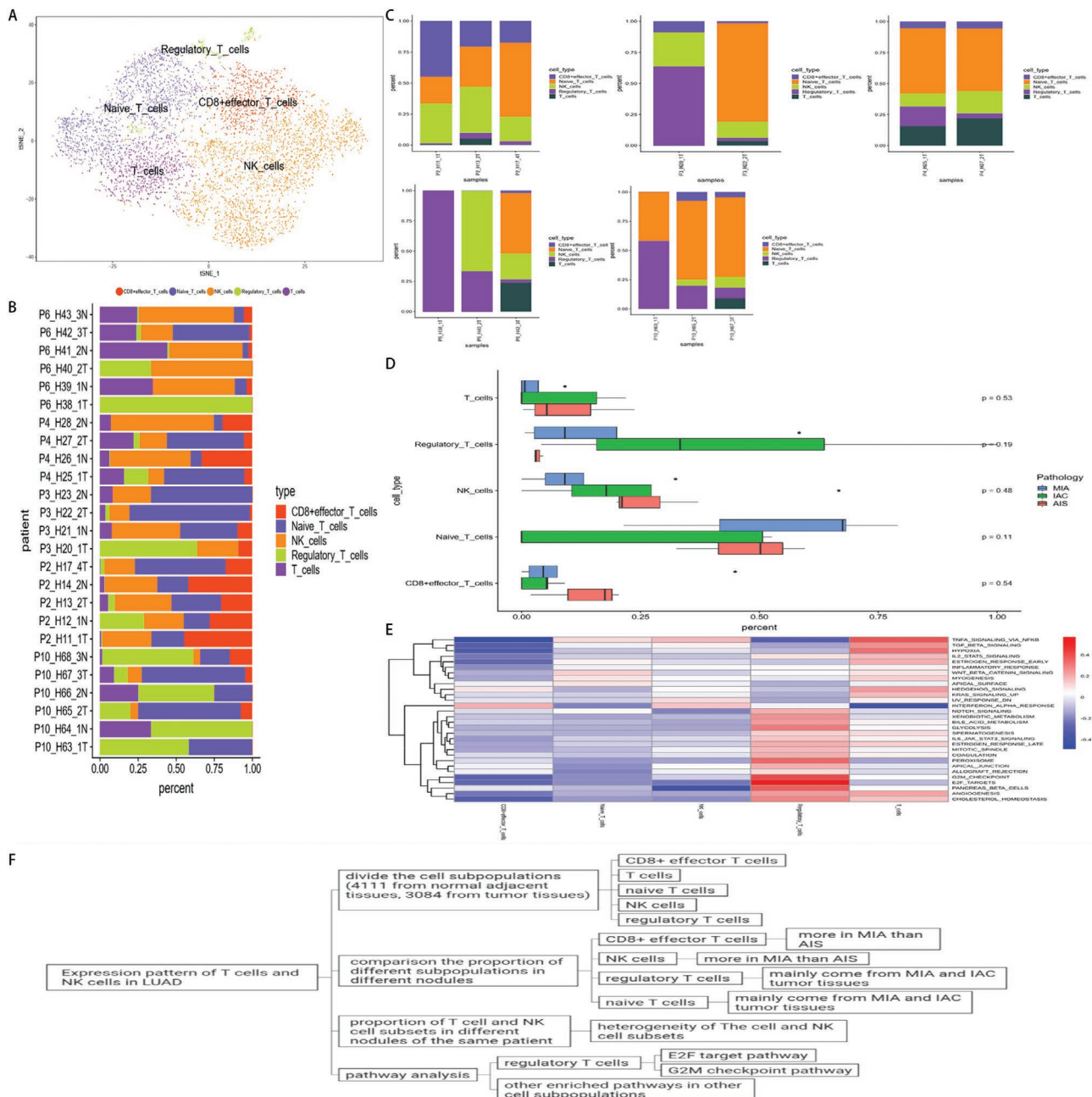


Figure 3. Expression pattern of T and NK cells in LUAD. A) tSNE plot of T cells, color-coded by cell subtypes. B) Fraction of cells originating from each of the five patients in T cell subtypes. C) Fractions of T cells originating from different nodules of the same patient. D) Expression of different T cell subtypes in MIA, IAC, and AIS. E) The pathway analysis of differentially expressed genes of T cell subsets. F) Flow chart of expression pattern of T and NK cells in LUAD.

response, coagulation, bile acid, xenobiotic, and fatty acid metabolism and adipogenesis pathways were enriched in the macrophage subset. The expression of the interleukin 1 beta (IL-1 β) gene in the subgroup of cDC2s is significantly higher than that of other subgroups, and this gene frequently appears in the above enrichment pathways (Figure 4G). Lung cancer incidence and survival rate are related to various inflammation-related factors, such as the IL-1 β .^[26] IL-1 β is highly expressed in the bigger paracancerous tissues corresponding to MIA and

IAC nodules, but in P4, it is highly expressed in the two nodules, not paracancerous (Figure 4G). IL-1 β induced MUC5B and MUC5AC protein secretion and mucus hyperconcentration in human bronchial epithelial cells.^[27]

To sum up, differential genes of DCs increased in the NF- κ B pathway and so on. In macrophages subsets, interferon-alpha response, coagulation, and other pathways were increased. cDC2s, monocytes, and pDCs were all mainly from normal tissues and worked in tumor invasion, which induced

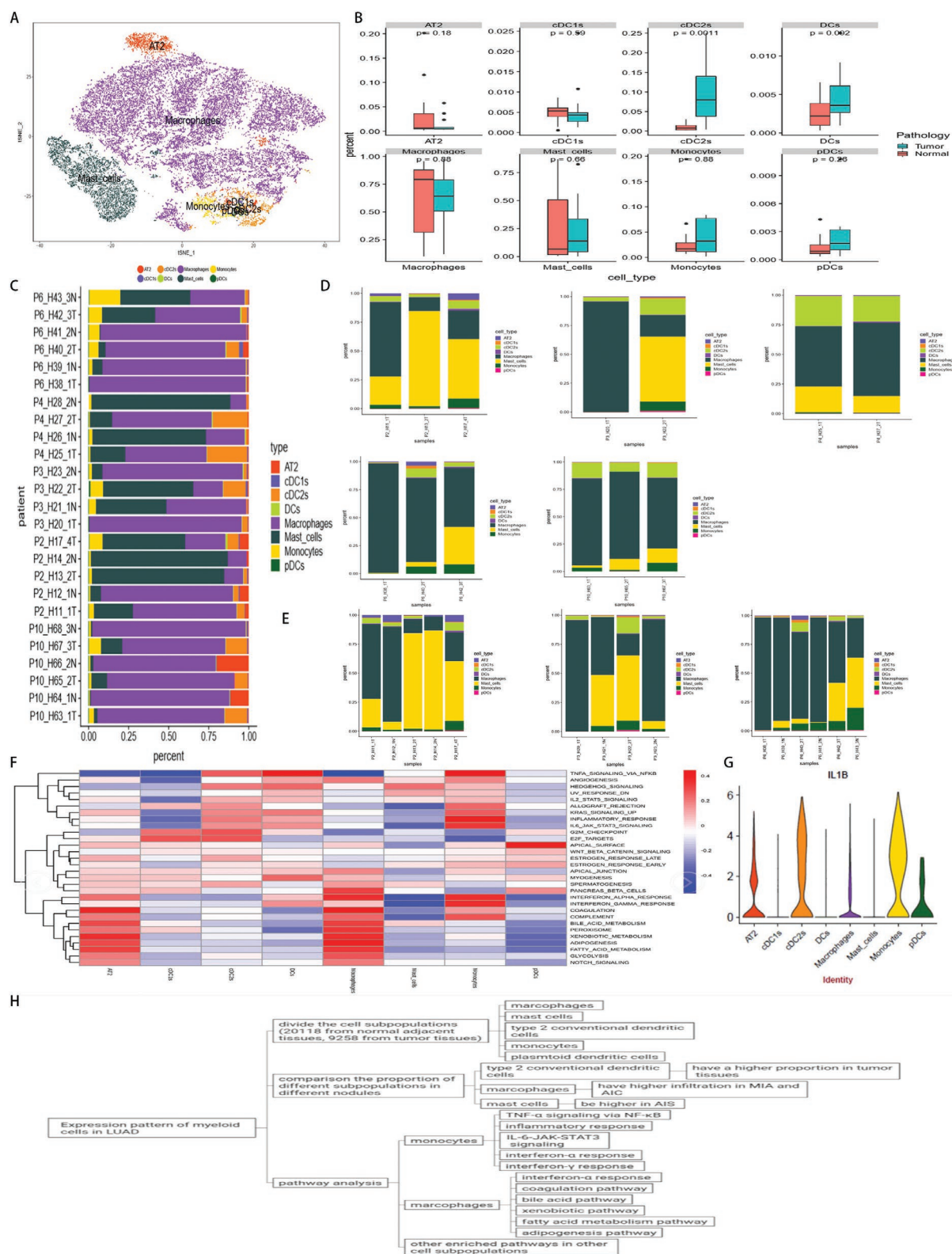


Figure 4. Expression pattern of myeloid cells in LUAD. A) t-SNE plot of myeloid, color-coded by the cell subtypes. B) Bar chart of the proportion of myeloid cell subtypes in normal and cancer tissues. C) The fraction of cells originating from each of the five patients in myeloid subtypes. D) The fractions of myeloid cells originating from different nodules of the same patient. E) The fractions of myeloid cell subtypes originating from NO.2, NO.3, and NO.6 patient. F) The pathway analysis of differentially expressed genes of myeloid cell subsets. G) The expression of genes on the myeloid subgroups and patients. H) Flow chart of expression pattern of myeloid cells in LUAD.

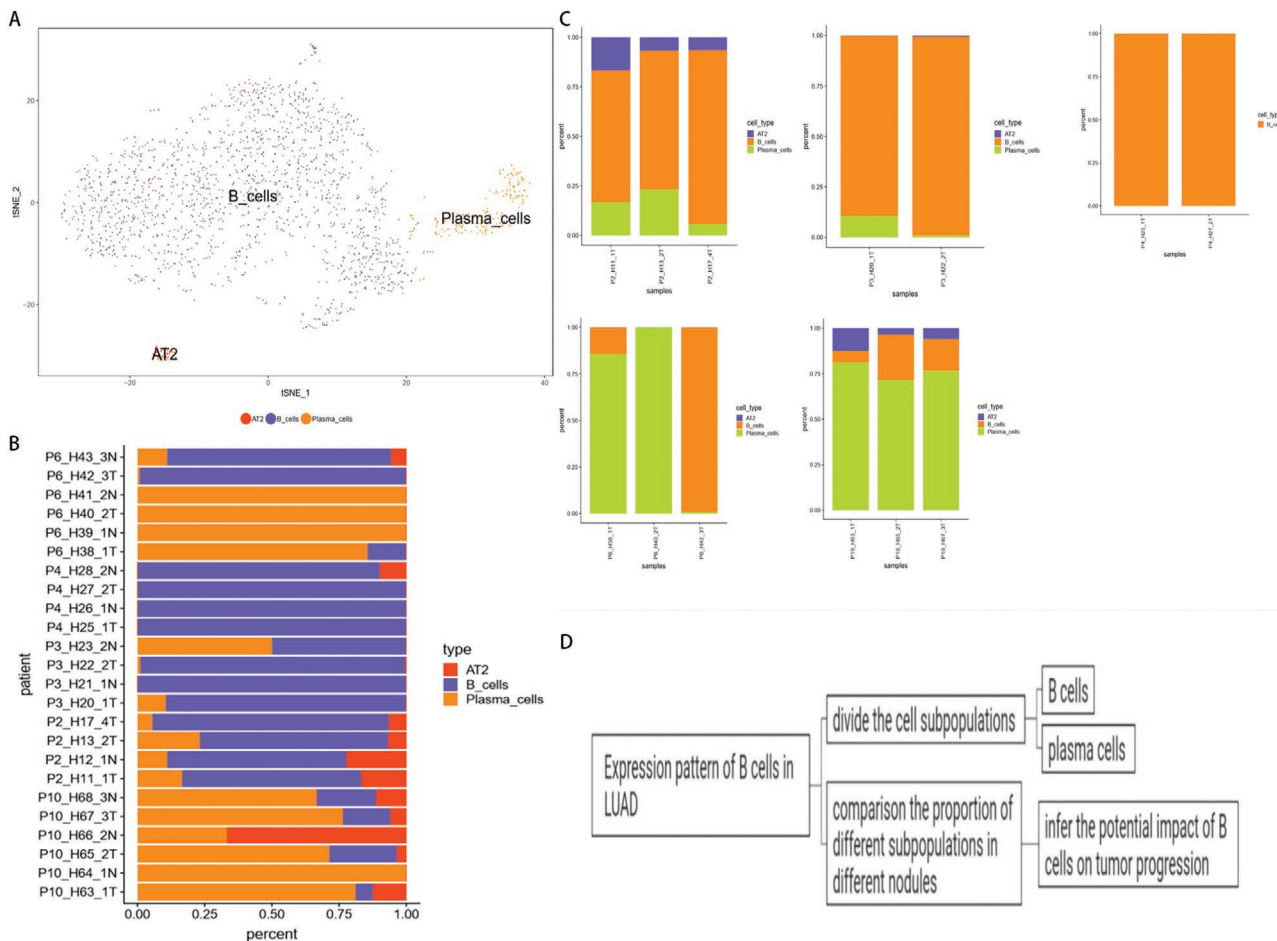


Figure 5. Expression pattern of B cells in LUAD. A) t-SNE plot of B cell, color-coded by the B cell subtypes. B) The fraction of cells originating from each of the five patients in B cell subtypes. C) The fractions of B cells originating from different nodules of the same patient. D) Flow chart of expression pattern of B cells in LUAD.

tumor progression and then changed tumor heterogeneity indirectly (Figure 4G). Although pDCs appeared to be fully developed when they left the bone marrow, they were relatively inefficient at presenting antigens to T cells and seemed to play an essential role in inducing tolerance, possibly by inducing regulatory T cells.^[28,29] pDCs are potent enhancers of the cDCs' capacity to prime antigen-specific CD8⁺ T cells responses, through which can link innate immunity and adaptive immunity.^[30,31] It has been reported that pDCs from human NSCLC tissues produce pro-angiogenic and invasive cytokines, such as IL-1 α , a cytokine with both tumor-promoting and anti-tumor effects.^[30,32] The flow chart was in Figure 4H.

2.2.3. Expression Pattern of B Cells in LUAD

We evaluated the expression characteristics of B cells in different patients and annotated three cell subpopulations. They were B cells, plasma cells, and AT2 (Figure 5A).

We analyzed the fraction of cells originating from each of the five patients in B cell subtypes and different nodules

of the same patient (Figure 5B,C). B cells were strongly enriched in tumors. The primary cell subsets of the nodules and adjacent tissues of P2, P3, and P4 patients were B cells (Figure 5B). The cell subpopulations of the two nodules H38 and H40 (both IAC) and adjacent tissues of P6 patients were mainly plasma cells, while H42 (AIS) of this patient was B cells. There was no significant difference between cancer and adjacent tissues except P3 H22 and H23, P10 H65, and H66 (both MIA).

The proportion of B-cell subtypes in different nodules in the same patient was lower, except for the P6 patient who was the only one with a history of smoking (Figure 5C). There were no regular patterns of B cell subtypes in different stages of nodules in different patients. While the two IACs of P6 were mainly plasma cell subgroups, the AIS nodule was mainly B cell subtypes (Figure 5C). It has been reported that the plasma cell subtype in LUAD was an independent negative prognostic factor, and might be one of the primary producers of the immunosuppressive cytokine IL-35.^[33]

To sum up, B cells could decrease tumor invasion, change the pathological status, and change tumor heterogeneity indirectly. The flow chart was in Figure 5D.

2.2.4. Explore the Correlation between the Evolution and Heterogeneity of LUAD and the Expression Pattern of Cancer Cells in LUAD

To further understand the heterogeneity of tumor cells, we extracted 8228 tumor cells and further divided them into 14 subgroups (Figure 6A). From the fraction of cancer cells originating from different tumor tissues of five patients, we found that cluster 0 was the primary cell subtype in P3 H20. Cluster 1–4 cells mostly came from P6 H38 and H40 (both IAC). Cells from clusters 5–7 were mainly from P6 H42 and P10 H63, H65, and H67. Cluster 8 cells came from P2, P3 (H22), and P4 (Figure 6B).

We also analyzed the proportion of tumor cell subsets in different nodules of the same patient and found that tumor cells derived from P2 and P4 were mainly in cluster 8 (Figure 6C). The composition of tumor cells in P3, P6, and P10 was different. Overall, nodules in P2 and P4 had low heterogeneity of different nodules in the same patient, while the heterogeneity in P3 and P6 were high. We speculated that the low heterogeneity of different nodules of P2 and P4 might be that the origin of the nodules was the same. Reasons for the high heterogeneity of P3, P6, and P10 might be that their cancer cells came from different sources and developed independently. And smoking might be another reason that caused high heterogeneity in cancer cells.

From the top marker gene of the heat map, the genes that were significantly up-regulated in cancer cell clusters were CCT2, CPM, Keratin 81, and Claudin 2. Literature pointed out that these genes all played essential roles in the development of lung cancer. It has been reported that higher levels of CCT2 in tumors correlated with reduced survival rate of lung cancer patients.^[34] Upregulation of the expression of carboxypeptidase M (CPM) has been shown in lipid pneumonia and could indicate lung tissue response to external stimuli.^[35] Keratin's expression in tumor tissues is significantly higher than non-malignant, suggesting that it may have a carcinogenic effect.^[36] Moreover, Claudin is highly expressed in LUAD and is involved in the promotion of cancer cell proliferation.^[37,38]

We also performed GSVA and GSEA analyses of each cluster. We found that the differentially expressed genes were significantly enriched in the following pathways: Notch signaling, Glycolysis, Hypoxia, and Hedgehog signaling (Figure 6D). In this study, the GSEA showed significant positive enrichment in E2F targets, hypoxia, inflammatory response, P53 pathway, and TNF- α signaling via NF- κ B (Figure 6E). From the expression of genes on the Notch signaling pathway in cancer subgroups (Figure 6F),^[39,40] we could see that the gene Hes1 was expressed in clusters 2, 3, and 4, which mainly came from P6, two IAC nodules H38 and H40, and P6 was the only smoking person. The expression of immune markers (MUC1, NKX2-1, NAPS, KRT7, CEA, SFTPA1, and SFTPB) was shown in Figure 6G. Except for SFTPA1 and CEA, the expression of immune markers in each tumor subgroup is relatively high.

Increasing evidence suggests that tumor cells can exhibit stem cell-like properties ranging from signature gene expression profiles to experimentally proven long-term self-renewal and reproduction capabilities.^[41] From Figure 6H, we could see

that the stemness score was related to the size of the nodules. The nodules of stemness score > 0 were H63, H65, H38, H40, and H20, which were the biggest in P10, P6, and P3. As there was no stem cell-like property of P2 and P4 nodules which had low heterogeneity, we hypothesized that the stemness score was related to nodular heterogeneity and the higher the stemness score was, the higher the heterogeneous of the nodules in the same patient (Figure 6C,H).

To sum up, heterogeneity did exist in all nodules, but the level of heterogeneity was different. Furthermore, the heterogeneity in the patient with smoking history was the highest in our study. Moreover, we found heterogeneity of the nodule correlated to the stemness that the tumor cells showed. Up-regulated genes were also detected in different cancer cells and pathways they enriched in, such as the NOTCH pathway, hypoxia pathway, Hedgehog signaling pathway, and so on.

2.3. Trajectories of the Tissues Revealed the Pathological Subtypes and Progression of LUAD

A complex process of cellular interactions accompanies the genesis and development of LUAD. Single-cell sequencing can help to study the process of cell dynamics, especially cell cycle, cell differentiation, and cell activation. These dynamic processes can be calculated and simulated by the trajectory information method. First, we used the unsupervised monocle 2.8.0 to construct the potential developmental trajectories of tumor cells (Figure 7). According to the results of the pseudo-time analysis, we found that the differentiation from the left to the right end of the branch showed the time from front to back (Figure 7A,D,G).

The samples in this research were derived from different nodules and adjacent tissues of five LUAD patients. We separately extracted the tumor cells from each patient and performed trajectory analysis. From the trajectory analysis of multiple clinical samples at different stages of the patient (Figure 7B,E,H), we were able to assist in determining the staging and progression of the tumor (Figure 7C,F,I). From the Figure 7A,B,C, we could see the developmental trajectory of epithelial cells in P3, from normal tissues (H23) to MIA tumors (H22) and IAC tissues (H20). In P6, the developmental trajectory started from the normal tissues to two different branches, smaller IAC nodule H40 or the bigger IAC nodule H38 (Figure 7D,E,F). In the P10, the developmental trajectory started from normal to the smallest MIA nodule H67, the more prominent MIA nodules H63 and H65 (Figure 7G,H,I). From the perspective of the developmental relationship, the origin of different nodules of P3 and P10 may be the same, while the source of different nodules of P6 may be different, probably because of the smoking history of P6.

3. Discussion

In this study, we presented a comprehensive catalog of the tumor and stromal cells in human LUAD and adjacent tissues at single-cell resolution. In describing key molecular differences between nodules and normal cells co-opted by tumors

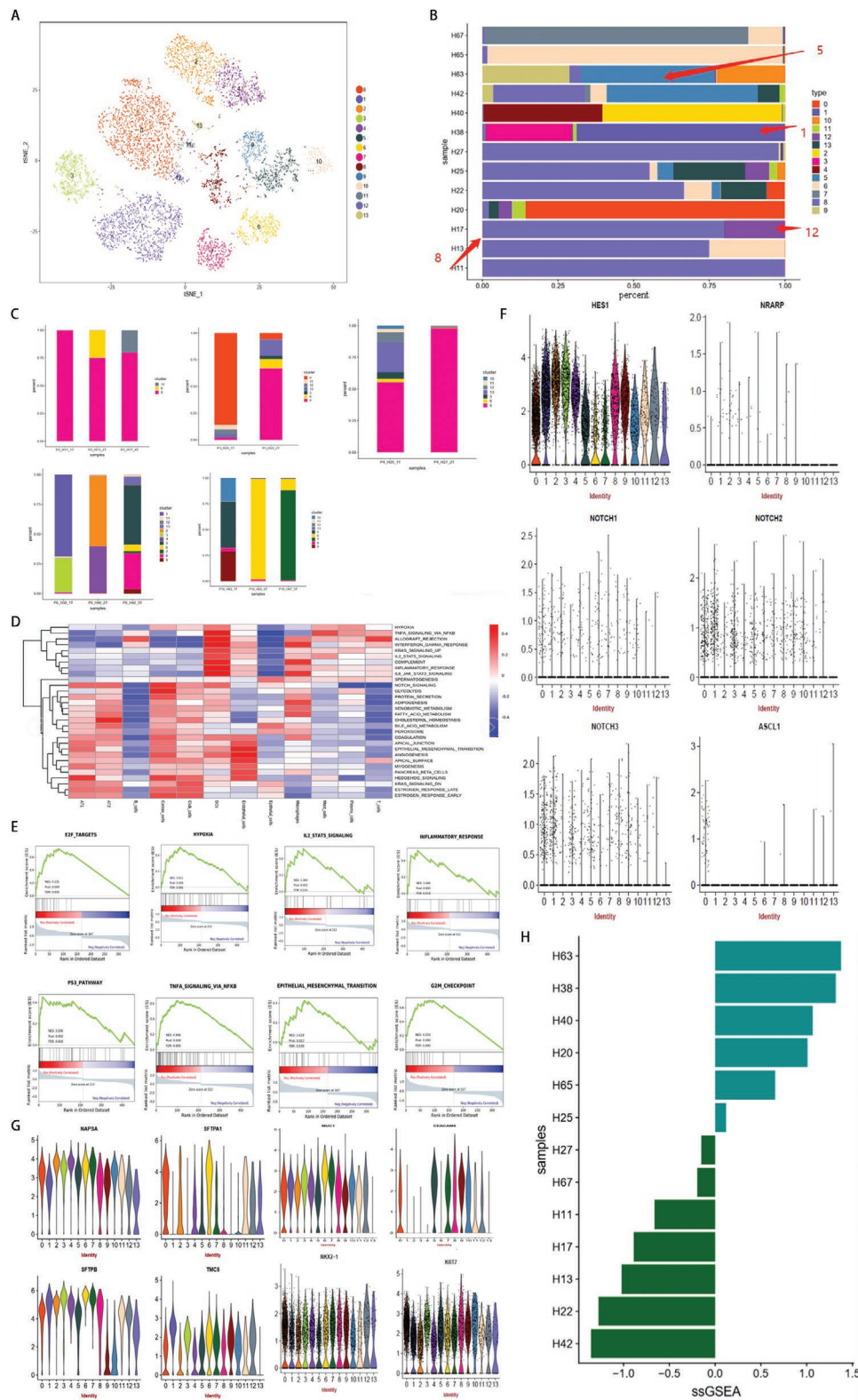


Figure 6. Expression pattern of cancer cells in LUAD. A) t-SNE plot of cancer cells, color-coded by their associated clusters. B) Fractions of cancer cells originating from different nodules of the same patient. C) Fraction of cells originating from each of the five patients in cancer subtypes. D) Heatmap shows genes that are differentially expressed across 14 clusters of cancer cells. E) The pathway analysis of differentially expressed genes of five patients. F) The GSEA analysis of differentially expressed genes. G) The expression of genes on the NOTCH signaling pathway in cancer cell subgroups. H) The gene expression of LUAD biomarkers. I) The stemness score of different nodules.

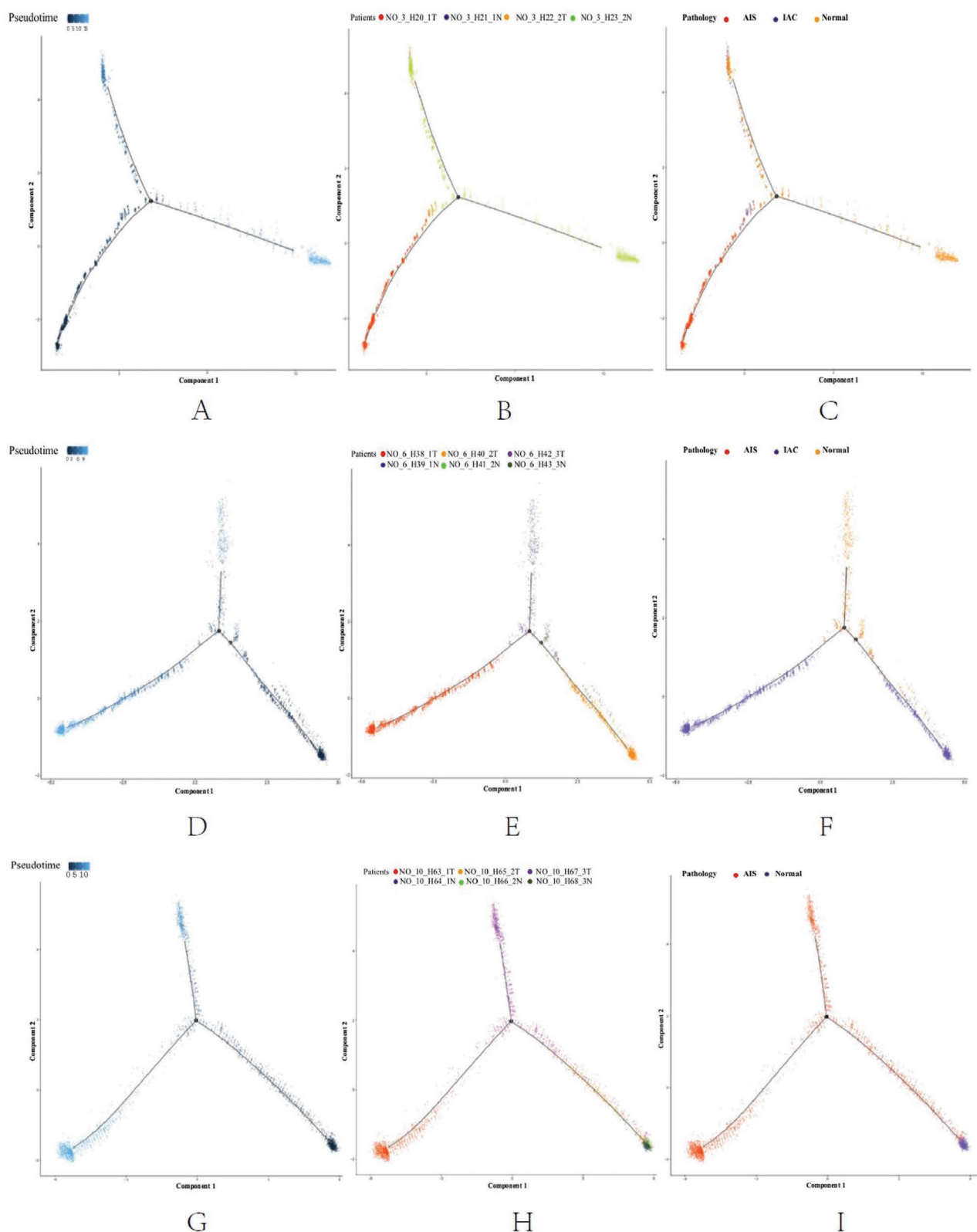


Figure 7. Trajectories of the tissues revealed the staging and progression of LUAD. A,D,G) The pseudotime trajectory of LUAD cell state transition. B,E,H) Development trajectory of different samples. C,F,I) The branched trajectory of LUAD cell state transition in a 2D state-space inferred by Monocle, and in P3 (B), P6 (E), and P10 (H).

and adjacent tissues, our analyses confirmed many valuable observations and highlighted key points for further advances in nodules of early LUAD. By identifying cell subtypes and enriching pathways, cataloging marker genes, analysis of heterogeneity of different nodules, and trajectory analysis, this dataset would fuel advances in heterogeneity and evolution of LUAD. As the dataset contained characteristics of the composition of stromal cells and tumor cells as well as their molecular characteristics from different pathological subtypes of LUAD simultaneously, we showed the changes in LUAD during tumor progression from AIS to IAC. These findings could improve the understanding of the development of LUAD and show some potential therapeutic strategies which deserved further validation.

Although all cell types, subtypes, and phenotypes could not be fully described here, some key observations emerged. First, more than 50% of plasma cells, endothelial cells, DCs, and B cells came from tumor tissues; more than 50% of T cells, mast cells, macrophages, club cells and alveolar epithelial type 1 and type 2 cells came from adjacent normal tissues (Figure 2A–C).

Second, as cancer stromal cells were essential parts that had an impact on cancer cells' biological behavior in cancer, we also analyzed the cancer stromal cells. The highest correlation coefficient was found between LUAD and myeloid cells and alveolar cells and myeloid cells through single-cell transcriptomic network analysis. We also found that the differential gene could influence some pathways such as the Notch signaling pathway, hypoxia pathway, P53 pathway, TNF- α signaling via NF- κ B, and more. Recent studies had shown that Notch signaling was associated with drug resistance. In both LUAD tissues and resistant cell lines, NOTCH 3 was up-regulated.^[42] Notch signaling pathway played an important role in lung cancer initiation and cross-talks with several transcriptional factors, mediates cell fate decisions, tumor-suppressive or carcinogenic depending on the specific situation.^[43,44] Pro-inflammatory cytokine, tumor necrosis factor α regulates inflammatory and immune responses by up-regulating gene expression in a manner that is dependent on the transcription factor NF- κ B. In our study, the decrease of NF- κ B, inflammatory response, allograft rejection, IL6/JAK/STAT3 signaling, and the increase of the coagulation pathway, the hedgehog pathway could work on different cells. All these cells could be divided into two groups, one mainly came from tumor tissues, and another mainly came from normal tissues. The first group's cells had a common characteristic: they tended to have a large number in IAC compared with AIS and MIA, while the cells in the latter have the opposite tendency. It implied that the first group affected to promote tumor invasion.

Meanwhile, with the pathological subtypes coming from AIS to IAC, higher heterogeneity appeared. In our study, we found that CD8⁺ effector T cells, NK cells, and myeloid cells were facilitating factors to the high heterogeneity of tumors, and these cells mainly came from normal tissue. Since B cells were restraining factors to heterogeneity, further studies are needed to find how these stromal cells influence the heterogeneity of tumors, which will help us to find new therapeutic targets.

Third, factors in cancer cells could lead to the heterogeneity of nodules as well. In early samples of LUAD, where the proportion of tumor cells was lower than 30% of all cells in tumor tissues, heterogeneity was high between the different nodules

and between the nodules and the corresponding adjacent tissues in patients P3 and P6 (Figures 2E and 3B,C). From the top marker gene of the heat map, the genes that were significantly up-regulated in clusters were *CCT2*, *CPM*, *KRT81*, and more (Figure 6D). It had been found that a higher level of *CCT2* from tumors would reduce survival rates in patients with lung cancer.^[34] The high *CPM* activity might be due to its induction and release in acute lung disease.^[35] Meanwhile, the expression of *KRT81* in tumor tissues was significantly higher than that of non-malignant, suggesting that it might have a carcinogenic effect in canceration.^[36] High expression of surfactant genes (*SFTPA1*, *SFTPA2*, and *SFTPC*) was strongly associated with death on a ventilator and type II pneumocyte hyperplasia. Dynein (*DNAH9* and *DNAH12*) and mucin (*MUC5B* and *MUC16*) genes are exclusive to the respiratory epithelium and goblet cells of bronchial structures.^[45] The high expression of *MUC5B* was significantly associated with poor outcomes in LUAD.^[46] *S100A9* was calcium and zinc-binding protein that played an essential role in regulating inflammatory processes and immune responses.^[47] *SAA1* and *CXCL14* promoted tumor cell growth and invasion.^[48] In normal lung tissues, *AZGP1* mRNA and protein expression were low or absent, while in LUAD they were highly expressed, which may be affected by chromatin remodeling utilizing histone acetylation.^[49] *CP* is a gene related to the stage of LUAD; its expression was significantly higher in IAC than in AIS and was significantly correlated with more unsatisfactory outcome.^[50] As we found the expression of these genes had changes in tumor tissue, it might suggest that they were involved in carcinogenesis and cancer progression. Further research is necessary for clearly understanding their function and may give us new thoughts for treatment. In addition, we also found that the stemness score was related to nodular heterogeneity and the higher the stemness score was, the higher heterogeneous of the nodules in the same patient. Cancer stemness contributing to tumor heterogeneity has also been reported and it plays important roles in malignancy metastasis, recurrence, and drug resistance.^[51] Previous studies also studied the relationship between cancer stemness and anti-tumor immunity and revealed that cancer stemness was negatively correlated with immune infiltration in LUAD.^[52,53]

What is more, we found that smoking might cause high heterogeneity in cancer cells (Figure 6B) because the only patient who smoked showed high heterogeneity, but it still needs to be proved.

Fourth, from the trajectory analysis of multiple clinical samples at different stages of the patient, we were able to assist in determining the evolution and progression of the tumor. We could also find the change of cells from the alveolar epithelial cells to different tumor cells in patients 3, 6, and 10, which showed tumor progression. However, in patients 2 and 4, there was no apparent developmental trajectory relationship. This data indicated that different patients might have a distinct carcinogenesis process.

Last but not the least, we found some changes during the development from AIS to IAC. For example, in Patient 6, compared to AIS nodule, the proportion of clusters 1 and 2 significantly increased in IAC nodule while the proportion of clusters 5 and 8 decreased. We also found the differences in gene expression among the clusters. These differentially expressed genes might influence the progression of LUAD, and targeting

them might have the ability to repress or even reverse the tumor progression. Differences in the tumor-stromal cell, such as a higher proportion of macrophages, were found in IAC. Higher infiltrating levels of NK cells in paired normal tissue of IAC were also detected between pathological progressions. Thus, by changing the infiltrating ratio of these cells or targeting their function, tumor progression might be controlled. Our finding uncovered the immune heterogeneity during tumor progression from multiple LUAD nodules and their associations with cancer evolution from AIS to IAC. With the pathological subtypes coming from AIS to IAC, higher heterogeneity appeared. We also investigated factors influencing the heterogeneity of nodules. These findings made us know more about LUAD evolution and might uncover some potentially new therapeutic targets to control tumor progression.

4. Experimental Section

Patients: Lung cancer nodules and adjacent tissues from five multiple nodules lung adenocarcinoma patients were analyzed. Patient information was presented as described in Table 1. This study was conducted following the provision of the Declaration of Helsinki and approved by the ethics committee of Shanghai Pulmonary Hospital (No. ys2018-003). Written informed consent was obtained from all participating subjects.

Tissue Dissociation and Single-Cell Suspension Preparation: Fresh tissue samples were collected and immediately stored in the GEXSCOPE Tissue Preservation Solution (Singleron Biotechnologies) at 2–8 °C. Before tissue dissociation, the specimens were washed with Hanks Balanced Salt Solution (HBSS) for three times and minced into 1–2 mm pieces. The tissue pieces were digested in 2 mL GEXSCOPE Tissue Dissociation Solution (Singleron Biotechnologies) at 37 °C for 15 min in a 15 mL centrifuge tube with continuous agitation. Following digestion, a 40-micron sterile strainer (Corning) was used to separate cells from cell debris and other impurities. The cells were centrifuged at 1000 rpm, 4 °C, for 5 min, and cell pellets were resuspended in 1 mL PBS (HyClone). To remove red blood cells, 2 mL GEXSCOPE Red Blood Cell Lysis Buffer (Singleron) was added to the cell suspension and incubated at 25 °C for 10 min. The mixture was then centrifuged at 1000 rpm for 5 min and the cell pellet resuspended in PBS. Cells were counted with the TC20 automated cell counter (Bio-Rad).

Single-Cell RNA Sequencing Library Preparation: The concentration of single-cell suspension was adjusted to 1×10^5 cells per mL in PBS. The single-cell suspension was then loaded onto a microfluidic chip (part of Singleron GEXSCOPE Single Cell RNAseq Kit, Singleron Biotechnologies) and single-cell RNA-seq libraries were constructed according to manufacturer's instructions (Singleron Biotechnologies). The resulting single-cell RNAseq libraries were sequenced on Illumina HiSeq X10 instruments with 150bp paired-end reads.

Bioinformatic Analysis: Raw reads were processed to generate gene expression profiles using an internal pipeline. Briefly, after filtering read one without poly T tails, cell barcode and UMI was extracted. Adapters and poly A tails were trimmed before read two was mapped to GRCh38 with ensemble version 92 gene annotations. Reads with the same cell barcode, UMI, and gene were grouped to calculate the number of UMIs per gene. The cell number was then determined based on the “knee” method. We used Seurat to perform clustering analysis and differential expression analysis to find marker genes per cluster.

After quality control, the authors obtained 55 713 cells, with an average gene number of 778, with an average UMI number of 1704. Principal component analysis was applied on genes variably expressed across all cells following gene expression normalization for reading depth and mitochondrial read counts. Subsequently, graph-based clustering was applied on the informative principle components ($n = 18$).

Dimensionality Reduction, Clustering, and Annotation: The authors used the R software package Seurat (<https://github.com/satijalab/seurat>) to

transform the expression matrix into Seurat objects and filter out cells with less than 200 genes. For the remaining 55 713 cells, Regress Out function was used in the Seurat package to realize linear regression, the Normalize Data function to normalize, and the Find Variable Genes function for highly variable genes. Then, the principal component analysis was carried out, and the Run TSNE function was used to reduce the dimension of the tsne. Finally, a total of 19 clusters were obtained. Cells were annotated in a semi-automatic way and 13 cell types were found.

Immune Cells Analysis: To further understand the relevant characteristics of immune cells, T cells, B cells, and myeloid cells were extracted from the integrated analysis data, analyzing these three cell types. Principal component analysis and dimensionality reduction were done on immune cells. The Find Clusters function in the Seurat package was used for clustering. The resolution parameter was set to 0.6, and the others were default parameters. Each cell type was reclustered with its principal components. ANOVA was used to compare three groups.

Identification of Marker Genes: In order to identify the marker genes of the immune cell subcluster, we used the Seurat Find Markers function to compare the subpopulation cells with other subpopulation cells to find the different genes. According to the list of differential genes found by Seurat and the expression of classical genes searched for in some subgroups manually, the cell subsets were annotated.

Gene Set Variation Analysis (GSVA): First of all, the Seurat V2.0 object was preprocessed, artificially increased five times for each cell type, randomly selected 100 cells for each repetition, calculated the average expression, analyzed the five average expression values with the software package GSVA (10.1186/1471-2105-14-7), and selected 50 hallmark pathway files for the gene set. Finally, the average value was calculated according to GSVA enrichment scores, and the map was drawn with heatmap.

Development Trajectory Analysis: Monocle 2.8.0 unsupervised method was applied to analyze tumor cells and alveolar cells of No.3, No.6, and No.10 in patients, effectively avoiding the influence of individual variation analysis results on all tumor cells. Each point represented a cell, colored according to each cluster cell type. Used the importCDS function in Monocle package to import the Seurat object into monocle, and used the new Cell Data Set function to build the cds object in a monocle. Used the detect Genes function to count the number of specific genes expressed. Used the setOrderingFilter function to mark the genes for subsequent clustering, the “DDRTree” method was used to reduce the dimension, and then used order Cells to sort the cells. Finally, the plot_cell_trajectory function was used to draw the trajectory analysis diagram.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Y.H. and X.L. contributed equally to this work. Conceptualization: Y.H., X.L., and Y.Y.; Methodology: Y.H. and X.L.; Software: Y.H., X.L., and J.Z.; Validation: L.W., J.Z., and Y.Y.; Formal Analysis: X.L. and Y.H.; Investigation: L.W., H.W., M.J., and H.G.; Resources: L.W., J.Z., H.S., S.C.,

and Y.Y.; Data Curation: Y.H., X.L., and Y.Y.; Writing – Original Draft: Y.H., X.L., H.W., M.J., H.G., and S.W.; Writing – Review & Editing: All authors; Visualization: Y.H., X.L., H.W., M.J., H.G., H.S., and S.C.; Supervision: Y.Y.; Project Administration: Y.H., X.L., and Y.Y.; and Funding Acquisition: Y.H.

Data Availability Statement

Research data are not shared.

Keywords

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